Extrasynaptic GABA Type A Receptors in the Mechanism of Action of Ethanol

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The γ-aminobutyric acid (GABA) Type A receptor ($GABA_A$-R) mediates the majority of rapid inhibition in the central nervous system and is the site of action for many clinically used drugs. $GABA_A$-R mediated inhibition can occur via the conventional mechanism - the transient activation of synaptic receptors i.e. phasic inhibition, or via continuous activation of extrasynaptic, high affinity receptors by low concentrations of ambient GABA, leading to "tonic" inhibition. The $GABA_A$-R α4 subunit is expressed at high levels in the dentate gyrus and thalamus and when partnered with the δ subunit, it is suspected to contribute to tonic inhibition. In vitro studies have found that $GABA_A$-Rs containing α4 and δ are highly sensitive to ethanol and to competitive $GABA_A$-R agonists such as gaboxadol and muscimol. In light of these findings, the central hypothesis tested in this thesis was that extrasynaptic $GABA_A$-Rs mediate the depressant effects of these drugs. To provide a model for understanding the precise role of α4. containing $GABA_A$-Rs in drug action, mice were engineered to lack the α4 subunit by targeted disruption of the *Gabra4* gene. α4 Subunit knockout mice were viable and superficially indistinguishable from wild-type mice. In electrophysiological recordings, α4 knockout mice showed a lack of tonic inhibition in dentate granule cells and thalamic relay neurons. α4 knockout mice were also less sensitive to the behavioral effects of gaboxadol and muscimol. However, α4 knockout mice did not differ in ethanol-induced changes in anxiety, locomotion,

ataxia, coordination, analgesia, or thermoregulation. These data demonstrate that tonic inhibition in dentate granule cells and thalamic relay neurons is mediated by extrasynaptic $GABA_A$-Rs containing the α4 subunit and that gaboxadol and muscimol likely achieve their effects via the activation of this $GABA_A$-R subtype. These data also suggest that $GABA_A$-Rs containing the α4 subunit are not necessary for many acute behavioral responses to ethanol.

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LIST OF ABBREVIATIONS

BEC	blood ethanol concentration
CNS	central nervous system
DGGC	dentate gyrus granule cells
ES	embryonic stem
EtOH	ethanol
GABA	γ-aminobutyric acid
$GABA_A$-R	γ-aminobutyric acid type A receptor
GBX	gaboxadol
i.p.	intraperitoneal
kb	kilobase
kDa	kilodalton
KO	knockout
LGIC	ligand-gated ion channel
LORR	loss of righting reflex
VB	ventrobasal
WT	wild type

1.0 INTRODUCTION

1.1 MOLECULAR MECHANISMS OF ETHANOL

Ethanol is the most frequently used and abused drug in our society. Ethanol-related accidents and other problems caused by alcohol abuse cause tremendous human suffering. Alcohol abuse has many long-term effects than can result in increased propensity for serious illness and premature death [1]. The abuse of this substance places an enormous financial burden on society; it has been estimated that the total cost of medical care and loss of productivity due to alcohol abuse is over 180 billion dollars per year in the United States, alone [2]. However, there is increasing evidence that controlled low to moderate ethanol consumption has a number of long-term positive health implications. These potential beneficial ethanol effects include lower rates of heart attack, reduced heart failure rate, and lower risk for dementia [3]. In light of these important implications for humanity, a large effort of biomedical research is dedicated to ethanol-related topics.

Ethanol is a central nervous system (CNS) depressant. At low blood ethanol concentrations, there is a feeling of euphoria, disinhibition, and decreased anxiety. At slightly higher concentrations, motor function is impaired and speech becomes slurred. Still higher concentrations can produce stupor and hypnosis. While it is evident that ethanol affects the

CNS, our understanding of the precise molecular mechanisms by which ethanol achieves these effects remain unclear [4]. One reason for this gap in knowledge is that our understanding of the cellular and molecular events in the brain circuits that underlie these behaviors are still largely unknown. It is widely accepted that brain function can be ultimately explained by current flow across neuronal cell membranes. How this movement of charged particles results in awareness, sensation, motor control, cognitive functions and emotions has been the subject of decades of research. It is likely the outcome of a complex "puzzle" including electrochemical signal-transduction between neurons, spatial and temporal summation of currents and precise control of the neuronal network. It is unclear which parts of this puzzle ethanol disrupts to produce its effects on behavior. Many theories have been put forth but there is little consensus about specific mechanisms. For many years it was believed that ethanol produced its CNS effects through the non-specific disruption of neuronal lipid bilayers thereby preventing signal transduction between neurons. However, it is now generally accepted that instead, ethanol alters the function of specific proteins. Some of these protein targets include ion channels e.g., [5-8]; G-protein coupled receptors [9-12]; and a number of second messenger proteins, e.g. [13].

Ligand-gated ion channels (LGICs) appear to be an important class of proteins affected by ethanol. LGICs are membrane bound neurotransmitter receptors that are widely distributed in the mammalian CNS. These receptors are responsible for rapid neuronal transmission through synaptic transmission and for regulation of neuronal excitability. Different LGICs transmit either excitatory or inhibitory signals between neurons. Ethanol directly affects the activity of several LGICs including the inhibitory γ-aminobutyric acid type A ($GABA_A$) and glycine receptors as well as the excitatory, N-methyl-D-aspartate (NMDA), neuronal nicotinic acetylcholine and 5-hydroxytryptamine (serotonin) type 3 ($5\text{-}HT_3$) receptors [12, 14-18]. Ethanol

does not affect LGICs in a random fashion. Rather, the depressant effect of ethanol is consistent with its observed effects on LGICs. Ethanol inhibits excitatory neurotransmission mediated by NMDA receptors [18] and enhances inhibitory neurotransmission at $GABA_A$ receptors [16].

Ethanol clearly interacts with and modifies the function of a number of LGICs and other proteins. However, it is likely that some proteins play more important roles than others in mediating the effects of ethanol in the CNS [5]. There is a large body of evidence showing that ethanol enhances the effects of GABA, the major inhibitory neurotransmitter in the CNS. To date, more than 4000 published studies describe a link between ethanol and GABA. GABA Type A receptors ($GABA_A$-Rs), the primary target of GABA, are one particular class of LGICs that may play a role in both the short and long term effects of ethanol in the CNS. The primary goal of this thesis is to further understand the contribution of specific $GABA_A$-R combinations to the molecular mechanisms of ethanol-induced behaviors.

1.2 THE GABA SYSTEM

1.2.1 Synthesis

GABA is the primary inhibitory neurotransmitter in the mammalian central nervous system. GABA is essential to neural activity, balancing the action of the predominantly excitatory neurotransmitter glutamate, which interestingly is a precursor of GABA. Through a process termed the GABA shunt, α-ketoglutarate is diverted from the Kreb's cycle and is converted to glutamate using GABA transaminase (GABA-T). Glutamate is converted to GABA by glutamate decarboxylase (GAD) of which two isozymes exist, GAD65 and GAD67.

Biosynthesis of GABA differs from that of most other neurotransmitters in that GABA precursors are part of the cellular intermediary metabolism of glucose rather than dedicated only to a neurotransmitter synthetic pool. In fact, 8-10% of overall brain glucose metabolism is funneled through the GABA shunt [19]. GABA is actively transported to the synaptic vesicles, where it is stored [20].

1.2.2 GABA release, reuptake and degradation

The vesicular release of GABA into the synaptic cleft occurs in a Ca^{+2}-dependent manner following depolarization of a GABAergic neuron. GABA release may be regulated by 1) autoreceptors for GABA located presynaptically; activation of these autoreceptors results in negative feedback and reduction of transmitter release or 2) hyperpolarization of the GABAergic neuron by another impinging GABAergic neuron. Reuptake is the primary mode of cessation of GABAergic transmission. GABA uptake by neurons and by glial cells takes place via one of at least four GABA transporters (GAT) [21]. The uptake is never complete, since 0.1 – 0.4 uM GABA can be detected in the extracellular space depending on the brain region [22, 23]. Degradation of GABA is carried out by GABA-T, the same enzyme involved in GABA synthesis. The products of GABA degradation are glutamate and succinic semialdehyde. While the latter feeds back into the Kreb's cycle, glutamate may be converted back to glutamine in glial cells and transported to neurons for subsequent use.

1.2.3 GABAergic neurons

It has been estimated that, depending on the brain region, up to 40% of brain synapses use GABA as their neurotransmitter [24]. Immunohistochemical detection of GAD and GABA receptors made it possible to map GABAergic neurons and their pathways in the brain. From extensive work using this method, it is obvious that GABAergic cells are found throughout the brain, and innervation is especially rich in the cerebral cortex, hippocampus, thalamus, substantia nigra, striatum and cerebellum [25]. GABA acts upon supraspinal interneurons and spinal interneurons involved in presynaptic inhibition. GABAergic neurons form hierarchical pathways comprised of projection or relay neurons and local circuits involving interneurons.

1.3 GABA RECEPTORS

1.3.1 Role of GABA receptors in disease and treatment

GABA plays an important role in many different behavioral and physiological mechanisms including locomotor activity, feeding behavior, aggression, sexual behavior, mood, regulation of pain sensitivity, cardiovascular regulation and thermoregulation [26]. Abnormalities in GABAergic signaling play a role in several disorders including anxiety disorders [27], sleep disorders [28], epilepsy [29], tremor [30], alcoholism [31], and schizophrenia [32]. For this reason, the GABA system has been targeted for development of drugs that treat these disorders. Most of the pharmacological manipulation of the GABA system has focused on agonizing the effects of GABA, in particular by enhancing the effects of GABA

at GABA receptors. GABA receptor agonists have a wide range of clinical uses such as anti-convulsants, anesthetics, anxiolytics, muscle relaxants, sedative/hypnotics and depressants. However, many GABAergic drugs have untoward side effects or high abuse potential that results in psychological and physical dependence. Therefore, the development of more selective drugs that produce the desired effect without unwanted side effects is under pursuit.

1.3.2 GABA Receptor Types

Early pharmacological data suggested that at least two different types of receptors for GABA exist [33]. The fast component of inhibitory postsynaptic potentials was selectively blocked by bicuculline but the slow component was selectively blocked by phaclofen [34]. Molecular cloning and heterologous expression of recombinant receptors established the current view of two basic types of GABA receptors. Receptors mediating the fast inhibitory component are type A ($GABA_A$) and the slow component are type B ($GABA_B$) receptors.

$GABA_A$ receptors are members of the "cys-loop" superfamily of LGICs [reviewed in [35]]. Other members of this group are neuronal nicotinic acetylcholine, glycine and 5-hydroxytryptamine Type 3 receptors. Cys-loop LGICs are heteropentameric structures where the five subunits are arranged around a central pore that is permeable to ions. Agonist binding causes a conformational change in the receptor complex and opening of the ion channel. $GABA_A$-Rs are permeable to the negatively charged chloride and bicarbonate ions and therefore upon channel opening, fast neuronal hyperpolarization (and in some instances depolarization) results. $GABA_A$-Rs are the main targets for most GABAergic drugs and the central topic of this thesis. Their properties and significance will be discussed below.

$GABA_B$ receptors are activated by baclofen and belong to the family of G-protein coupled receptors that have seven transmembrane domains. They are composed of a single polypeptide chain, but functionally they are coupled in dimeric units [reviewed in [36]]. GABA binding activates inhibitory G-proteins that cause inhibition of adenylyl cyclase and agonist-induced inositol triphosphate synthesis, inhibition of Ca^{2+} channels and activation of inwardly rectifying K^+ channels. $GABA_B$ receptors may be located either pre- or post-synaptically, the former causing inhibition of neurotransmitter release and the latter causing hyperpolarization of the postsynaptic membrane. Activation of $GABA_B$ receptors has long-lasting inhibitory effects, and it has been shown to contribute to several important phenomena, such as regulation of LTP [37] and rhythmic activity in the hippocampus [37]. Alterations in $GABA_B$ receptor mediated function have been detected in animal models of depression, epilepsy and addiction [reviewed in [36]]. Despite the widespread distribution in the brain and the possibilities to affect behavior, the only FDA approved pharmacotherapy targeted specifically to $GABA_B$ receptors consists of baclofen treatment of spasticity associated with multiple sclerosis and spinal cord injury [38].

GABA Type C ($GABA_C$) receptors, like $GABA_A$ receptors, function as pentameric ligand-gated chloride ion channels. It has been suggested that $GABA_C$ receptors should be considered as a subgroup of $GABA_A$ receptors due to these similar structural features even though $GABA_C$ receptors are insensitive to the global $GABA_A$ receptor antagonist, bicucullline [39]. To date, three different subunits (ρ1-3) that comprise $GABA_C$ receptors have been cloned, and they can form homo- or hetero-oligomeric complexes. Additionally, these receptors are restricted mainly to the retina [reviewed in [40]] but may also be found in other brain regions [41].

1.4 $GABA_A$ RECEPTORS

1.4.1 Receptor subunits

All the members of Cys-loop superfamily are thought to possess a conserved tertiary and quaternary structure [42]. All of these receptors are comprised of five subunits arranged around a central pore. The subunits are 450-550 residue polypeptides in length. They all have a very large, extracellular N-terminal region containing agonist binding sites and the characteristic cysteine loop. All subunits contain four hydrophobic, putative membrane-spanning domains, where the second transmembrane (TM) domain forms the lining of the pore. A large intracellular domain between TM3 and TM4 is thought to contain phosphorylation sites and other regulatory domains. The C-terminus of each subunit protein is extracellular and very small compared to the N-terminal region.

A large number of subunits can comprise $GABA_A$-Rs; a total of nineteen distinct subunits have been cloned [43]. Subunits are grouped into seven classes based on homology. All classes are named by a Greek letter and some of the classes contain several isoforms. Thus far, α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3 have been identified [43-47]. Separate genes encode the different subunits, with some of the genes arranged in clusters on a particular chromosome.

Subunits typically display 70-90% sequence identity within a subunit class and 30-40% identity between classes [48]. In addition to numerous subunits, heterogeneity is increased by alternative exon splicing of the pre-mRNA. For example, there are two known splice variants of α6 [49] and β3, [50] and three of γ2 [51].

1.4.2 Spatial and temporal distribution of subunits

$GABA_A$-R subunits assemble into an undefined heterogeneous population of receptors that are unevenly distributed throughout the adult brain and vary throughout development [52, 53]. Distribution of subunits varies at both the cellular and subcellular level. For example, α1-containing receptors are localized primarily to interneurons and Purkinje cells, whereas α2 subunit-containing receptors are found on motor neurons and pyramidal cells [54]. α6 Subunit-containing receptors are found only in the cerebellum whereas α1 subunit-containing receptors are expressed throughout the brain [55]. Immunoctochemical studies have also revealed a distinct subcellular localization. For example, γ2 containing receptors are found at synaptic locations [56] while δ containing receptors are found almost exclusively extrasynaptically [57]. $GABA_A$-Rs are also expressed outside the CNS in the pituitary, adrenal medulla, pancreas and gut where they modulate the release of hormones and catecholamines and also regulate motility [58, 59].

There is also temporal control over subunit expression. $GABA_A$-R subunits can be plastic in expression. The expression of the α4 and α1 are dynamic and can be up or down regulated in a variety of pathophysiological situations [60-63]. Temporal and regional specific subunit expression patterns are observed throughout the developing brain [52, 64]. Alpha subunits have a temporal expression pattern in which α3, α5 and α2 and then α4 expression precede the predominant expression of α1 in the adult cerebral cortex [52].

1.4.3 Receptor subtypes in the brain

If there were no rules for receptor assembly, a theoretical maximum of the number of pentameric structures that could be made up from 19 different subunits would be 19^5 (~ 1 million). This number would be still further increased if alternative splicing of subunits were accounted for. However, there are strict, yet mostly unknown, rules that govern receptor assembly and therefore the number of subunit combinations is far less than the theoretical maximum. The exact number of $GABA_A$ receptor subtypes is unknown, however, more than twenty have positively been identified [65].

Several methods may be used to deduce receptor subunit composition. In situ hybridization can be used to localize different subunit mRNAs, and co-localization reveals the possibility of co-assembly [53]. Immunocytochemical studies have also revealed co-localization of multiple subunits in a single neuron, confirming the existence of a large variety of $GABA_A$-R subtypes in the brain [66, 67]. Still another technique utilizes antibodies to immunoprecipitate receptors containing a specific subunit from solubilized brain preparations. These can then be quantified using radioligand binding. This technique measures the abundance of each subunit even when present in several different receptor subtypes. By employing combinations of antisera it is possible to determine which subunits are co-assembled in the same receptor. Several limitations exist with all of these techniques and will not be comprehensively explained here. However, one of the primary obstacles has been the lack of high-affinity and high-specificity antibodies to many receptor subunits. As better subunit-specific antibodies become widely available, many more $GABA_A$-R subtypes will likely be discovered.

In vitro pharmacological studies using recombinant $GABA_A$-Rs have also contributed enormously to our knowledge, not only to the $GABA_A$-R subtypes that exist in the brain, but also

to the sensitivity and efficacy that different drugs have on various receptor subtypes. Different $GABA_A$-R subtypes can be artificially created and studied in cells that do not normally express $GABA_A$-Rs. For example, *Xenopus laevis* oocytes can be individually injected with subunit RNA purified from the brain [68], with synthetic subunit cRNAs [69] or with subunit cDNAs [70]. Another possibility for transient expression of $GABA_A$-Rs is to transfect cell lines (e.g., human embryonic kidney cells, mouse fibroblasts, or Chinese hamster ovary cells) with cDNA cocktails of different subunits [71]. These methods allow the study of a particular receptor population using electrophysiological recordings or binding studies. From recombinant receptor studies, it is possible to extrapolate those that are likely to exist in the brain based on their pharmacologic similarities. Drawbacks of this approach include poor expression of particular subunits *in vitro* [72] which may lead to large discrepancies in data acquired from different laboratories [73, 74]. Another major drawback to all recombinant receptor studies is that receptors are studied in a non-native environment. All of the cell types that are used lack neuron-specific intracellular proteins that interact with and perhaps, change the properties of $GABA_A$-Rs.

Despite these technical limitations, results from different techniques can be combined to identify some of the major subtypes present in the CNS (See **Table 1**). Most native receptors have a stoichiometry of two α subunits, two β subunits and one γ or δ subunit [75, 76]. It has been determined that the α1β2γ2 containing receptors is the one most commonly assembled [77] and has been estimated to comprise 43% of all $GABA_A$-Rs in the brain with the α2β3γ2 and α3β3γ2 subtypes accounting for another 35% of receptors [75]. Other subtypes include α6ββδ (~ 2 %, [57]) and α4ββδ (~ 1 %, [78]), which are less common, but highly important (discussed below).

Table 1. Major GABA(A) receptor subtypes in the brain.

Subunit combination	Relative abundance in rat brain (%)	Examples of brain regional localization	References
α1β2γ2	43	Present in most brain areas	Benke et al. 1991 Fritschy et al., 1992 Somogyi et al., 1996
α2β2/3γ2	18	Spinal cord motor neurons hippocampal pyramidal cells	Benke et al, 1994 Fritschy et al., 1998
α3β3γ2/3	17	Cholinergic and monoaminergic neurons	Fritschy et al., 1992 Quirk et al. 1994
α2β2/3γ1	8	Bergmann glia, nuclei of limbic system	Quirk et al. 1994
α5β3γ2/3	4	Hippocampal pyramidal cells	McKernan et al., 1991 Fritschy and Mohler, 1995
α6βγ2	2	Cerebellar granule cells	Quirk et al., 1994 Nusser et al., 1998
α6βδ	2	Cerebellar granule cells	Quirk et al., 1994 Nusser et al., 1998
α4β	2	Thalamus and hippocampal dentate gyrus	Bencsits et al., 1999
α4βγ2	2	Thalamus and hippocampal dentate gyrus	Bencsits et al., 1999
α4βδ	1	Thalamus and hippocampal dentate gyrus	Bencsits et al., 1999

1.5 $GABA_A$ RECEPTOR FUNCTION

Our knowledge of $GABA_A$-R activation is partially derived from our knowledge of another cys-loop LGIC, the nicotinic acetylcholine receptors. Binding of an agonist to nAChR

causes a small rotation of extracellular domains of subunits, which opens the channel formed by TM2 regions of adjacent subunits [79]. It has been demonstrated that $GABA_A$-Rs may function similarly; in recombinant receptors the α and β subunits rotate asymmetrically after GABA binding, which causes the channel to open [80]. When the $GABA_A$-R-associated channel is opened, various negatively charged ions may pass through it in both directions [81]. The primary anion passing through the channel is chloride, however, channels are permeable to many other anions including bicarbonate [82].

The overwhelming majority of $GABA_A$-R channel openings result in chloride influx into the cell. This is due to a favorable electrochemical gradient; in most CNS neurons, the chloride ion concentration is far greater in the extracellular environment than the intracellular. The influx of negatively charged ions results in hyperpolarization of the already negative cell membrane potential. For this reason, $GABA_A$-R activity results in neuronal inhibition. However, in some cells, $GABA_A$-R activation may cause depolarization due to increased intracellular chloride concentrations. This has been observed in the developing brain [83] and in primary afferent neurons [84].

Two main types of inhibitory neurotransmission are mediated via $GABA_A$-Rs [85, 86]. Synaptic ("phasic") inhibition results from the activation of receptors at the synapse by intermittent release of high concentrations of GABA from presynaptic terminals. In contrast, extrasynaptic ("tonic") inhibition is mediated by the continuous activation of receptors located outside the synaptic cleft by low concentrations of ambient GABA.

1.5.1 Phasic inhibition

The vast majority of $GABA_A$-Rs mediate inhibition via synapses in the brain. Phasic inhibition follows the classical model of neurotransmission and is crucial for normal brain function as it mediates the majority of cell to cell inhibition. An action potential in the pre-synaptic neuron causes vesicular release of GABA into the synaptic cleft, which activates $GABA_A$-Rs on the post-synaptic membrane. Many synaptic receptors are activated simultaneously, which causes a rapidly activating inhibitory postsynaptic current (IPSC) and hyperolarization of the postsynaptic cell. The IPSC is terminated within milliseconds when GABA is eliminated from the synapse by diffusion and uptake by transporters into neurons and glia [87]. Another factor contributing to the short duration of IPSCs is the rapid receptor desensitization caused by high agonist concentrations [88]. During desensitization, the agonist is still bound to the receptor, but the channel enters a closed state. Desensitization of synaptic $GABA_A$-Rs is necessary due to the high GABA concentration achieved in synapses [89]. GABA concentration in the synapse may rise to 300 uM or 3mM [90], whereas the concentration producing a half maximal effect (EC50) for the $GABA_A$ receptor subyptes is usually below 50uM [91].

1.5.2 $GABA_A$-R subtypes in phasic inhibition

IPSCs are made possible by the extremely high density of $GABA_A$-Rs in the post-synaptic membrane. How receptors are enriched in synaptic membranes is not fully known. Gephyrin is a 93 kD protein that anchors glycine receptors to synapses through its interaction with the cytoskeleton [92]. Substantial evidence suggests that gephyrin is also critical for

$GABA_A$-R clustering. While direct molecular interaction between gephyrin and $GABA_A$-R subunits has not been shown, gephyrin-deficient mice show decreased clustering of major $GABA_A$-R subtypes [93]. Gephyrin can be found co-localized with the γ2 subunit of the $GABA_A$-R as well as other receptor subunits that form functional receptors with γ2 such as α1, α2 and α3 [94]. The γ2 subunit is clearly important for synaptic anchoring; in γ2 knockout mice, postsynaptic $GABA_A$-R clusters are dramatically decreased and gephyrin clusters are also abolished [56]. However, overexpression of γ3 in γ2 knockout mice is sufficient to restore synaptic receptor clustering [95], which suggests that other γ subunits are also capable of interacting with anchoring proteins. GABA-RAP is another protein possibly involved in $GABA_A$-R clustering, and it has been shown to interact directly with the γ2 subunit [96]. It should be noted, however, that some γ2 subunit containing receptors are found in non-synaptic sites (e.g. α5βγ2 [97]. Nevertheless, it is clear that $GABA_A$-Rs containing the γ2 subunit (e.g. α1β2γ2, α2β3γ2 and α3β3γ2) play an important role in mediating synaptic inhibition [94].

1.5.3 Tonic inhibition

In addition to classical synaptic transmission, $GABA_A$-Rs also exert their actions by tonic inhibition, which is a phenomenon common to other neurotransmitter receptors (reviewed in [98]). Tonic inhibition is mediated by the continuous activation of $GABA_A$-Rs located outside the synaptic cleft by low concentrations of ambient GABA. Tonic inhibition results from the inability of GABA transporters to lower extracellular GABA concentration below 0.1 – 0.4 μM,

thereby keeping GABA receptors in permanent agonist bath of > 0.1 μM [23]. Extracellular GABA may originally have been spilled-over from neighboring synapses [23, 99]. Non-vesicular release of GABA from astrocytes or neurons is also possible [100, 101], which might be due to reversal of the GABA transporters [102]. Thus, the functional state of the transporters seems to be important in regulating tonic inhibition [23, 103]. Regardless, the presence of permanent agonist stimulation results in continuous receptor activation causing tonic inhibition.

Birnir and colleagues first identified $GABA_A$-R mediated tonic inhibition in the rat dentate gyrus in 1994 [104]. Thereafter it was found and characterized in the cerebellar granule layer [105, 106], the hippocampus CA1 region, [107, 108], dentate gyrus [109, 110], the cerebral cortex [109] and the thalamus [111]. The contribution of tonic conductance to the total inhibitory charge transfer varies depending on the brain region, but in the cerebellar granule cell layer, the vast majority of the inhibition is tonic in nature [106]. The exact functional roles of the tonic inhibition are unknown, but it is suggested to set a background level for overall neuronal excitability (reviewed in [112]). Tonic inhibition also has been suggested to control the synaptic responses by regulating general membrane properties [113]. In the cerebellar granule cells, tonic inhibition is shown to decrease the number of excited cells, which is suggested to improve information storage capacity [114]. In the hippocampus, tonic inhibition may affect learning and memory processes [115].

1.5.4 Receptor subunits involved in tonic inhibition

A clear distinction between the receptor subtypes responsible for phasic and tonic conductances is difficult to make, but some general points can be drawn. Tonic conductance may be derived from receptors located on both synaptic and extrasynaptic membranes, but owing

to receptor properties, extrasynaptic receptors are primarily activated. Since ambient GABA concentrations are maximally in a low micromolar range, the receptor subtypes responsible for tonic inhibition need to have a high GABA affinity. In addition, despite the persistent presence of an agonist, the receptors must not desensitize, otherwise they would be nonconducting. Taking these considerations into account, as well as results from genetically engineered animals, the roles that specific $GABA_A$-R subtypes play in tonic inhibition may be addressed.

Most convincing evidence indicates that δ subunit-containing receptors are responsible for tonic conductance in many brain regions. The δ subunit is almost exclusively extrasynaptic in its expression [57] and does not co-localize with gephyrin, the $GABA_A$-R synaptic clustering protein [94]. δ Subunit-containing receptors have an extremely high affinity for GABA (EC50 ~ 0.2-0.5 uM) [116-118] and desensitize poorly [119]. In addition, tonic conductance is greatly reduced in dentate gyrus granule cells of δ subunit knockout mice [109]. While δ containing $GABA_A$-Rs certainly contribute heavily to the tonic inhibitory current found in the brain, receptors devoid of δ might also play a role. For example, tonic inhibition in hippocampal CA1 pyramidal cells is greatly reduced in α5 knockout mice but these receptors are likely to be α5β3γ2 [97]. Additionally, functional receptors comprised of only α and β subunits exist [78]. Receptors comprised of α and β subunits have higher affinity for GABA [120, 121] and are located extrasynaptically, presumably due to a lack of γ2-gephyrin association [57].

In cerebellar granule cells, the α6 subunit protein is found on both synaptic and extrasynaptic membranes [57]. α6 containing receptors have high affinity to GABA [118], and they desensitize poorly [119]. Tonic current in cerebellar granule cells is detected after developmental induction of α6 subunit expression [106], and is blocked by the α6-specific antagonist furosemide [114]. In addition, tonic inhibition is abolished in the α6 knockout mice

[122]. As α6 is preferentially assembled with δ [123], most of the tonic conductance in cerebellar granule cells is probably mediated by α6β3δ receptors. However, given the pharmacological properties of α6β3γ2 receptors (e.g., ten-fold higher affinity for GABA than α1β3γ2 receptors; [118]), these receptors could also contribute to the tonic current.

The α4 subunit is expressed in the forebrain with particularly high expression in the thalamus and dentate gyrus. A recent study by Jia and colleagues observed a tonic inhibitory current on thalamic ventrobasal (VB) neurons using slice electrophysiology [111]. Immunohistochemical studies on VB neurons demonstrated co-localization of α4 and δ at extrasynaptic sites [111]. Like α6 in the cerebellum, α4 forms functional receptors with δ in the thalamus and dentate gyrus [78, 124]. Additionally, α4β3δ receptors, like their α6 containing counterparts have high affinity for GABA [116]. Therefore, it is likely that $GABA_A$-Rs containing the α4 subunit mediate tonic inhibition in the thalamus and dentate gyrus. This could be determined unequivocally by examining α4 knockout mice for the absence of a tonic inhibitory current.

1.6 $GABA_A$ RECEPTOR PHARMACOLOGY

1.6.1 General considerations

Several endogenous or exogenous agents act on $GABA_A$-Rs via several drug recognition sites. Agents that directly activate $GABA_A$-Rs by binding to the GABA site include endogenous GABA, as well as the exogenous ligands muscimol, and gaboxadol (these agents will be further discussed in later sections). None of these direct activating GABAmimetic drugs are currently

used in a therapeutic setting, however, the potential of gaboxadol as a sleep aid was explored [65]. Several agents enhance the effects of GABA without directly activating $GABA_A$-Rs; these include benzodiazepines, barbiturates and certain neuroactive steroids such as alphaxalone, pregnanalone and THDOC [125-127]. Of these, benzodiazepines are the most commonly prescribed class of GABAergic drugs and are useful in treating anxiety, insomnia, seizures, muscle spasms and alcohol withdrawal. Ethanol and general anesthetics are also GABAergic [128, 129] producing their effects through unknown mechanisms.

Bicuculline and SR 95531 are competitive GABA antagonists- they bind to GABA sites on $GABA_A$ receptors but produce no activation [130]. Non-competitive antagonists include picrotoxin, t-butylbicyclophosphorothionate (TBPS) and pentylenetetrazole (PTZ); these agents block the chloride channel and prevent ion flux [131]. $GABA_A$-R antagonists have no clinical usage thus far; administration of these agents can lead to hyperexcitability, seizures and eventually, death. Other negative modulators of $GABA_A$-Rs include β-carbolines, Ro15-4513 and the neurosteroid, pregnenolone [130] and are considered inverse agonists. Inverse agonists possess intrinsic activity but act in a manner directly opposite to agonists by attenuating the effects of GABA.

1.6.2 Benzodiazepines and $GABA_A$-R subtype specific interactions

In recent years it has been discovered that various GABA modulators do not affect all $GABA_A$-R subtypes equally. Studies investigating subtype selectivity of benzodiazepines provide a clear example. For example, only $GABA_A$-Rs containing either α1, α2, α3 or α5 in conjunction with the γ2 subunit are sensitive to the classical benzodiazepine, diazepam [132]. Interestingly, α1, α2, α3, and α5 subunits differ from α4 and α6 with respect to a single residue in

a highly conserved region of the extracellular domain (around the 100th amino acid) where the former contain a histidine residue and the latter an arginine residue. A histidine to arginine mutation in α1 makes receptors diazepam-insensitive, and conversely, an arginine to glutamine mutation in α6 makes these receptors diazepam sensitive [133].

Recent studies demonstrate that different $GABA_A$-R subtypes mediate the various behavioral effects of benzodiazepines. Genetically engineered mice with the aforementioned missense histidine to arginine mutation were created in either the α1 [134], α2 [135] or α3 [135] subunits rendering these $GABA_A$-R subtypes insensitive to diazepam. Phenotypic analyses of these point mutant mouse lines demonstrated that α1 containing $GABA_A$-Rs mediate sedation, anterograde amnesia, and seizure protection [134, 136], whereas α2-$GABA_A$ receptors, mediate anxiolysis [135]. These data provided a strong rationale for drug targeting to specific receptor subtypes and recently, it was reported that an α2 selective agonist produced anxiolysis without sedation in preclinical models [137].

These studies clearly demonstrate that $GABA_A$-R subtypes, through their specific regional, cellular and subcellular localization, are linked to distinct neuronal circuits and consequently serve distinct behavioral functions. Continued dissection of $GABA_A$-R subtypes using both recombinant receptor systems as well as genetically engineered mice will allow us to understand the function underlying other subypes with respect to other GABAergic drugs such as alcohol and other sedative hypnotic drugs. The elucidation of such mechanisms may provide the pharmacological basis for more effective treatments for alcoholism or for antagonists that can attenuate ethanol's adverse effects.

1.6.3 Pharmacology – Direct $GABA_A$ receptor agonists

The success of GABAergic drugs like benzodiazepines and barbiturates spurred development of new agents that activate $GABA_A$-Rs. One obvious strategy was to create drugs that mimicked the action of GABA itself. Muscimol, a constituent of the mushroom *Amanita muscaria* and structurally similar to GABA, primarily activates $GABA_A$-Rs [138]. The use of muscimol had been investigated for the treatment of chronic pain, anxiety, epilepsy, schizophrenia, and movement disorders, but its low bioavailability and strong side effects prevented clinical usage [138]. However, muscimol has been used extensively as a lead compound for the design of several other GABA analogs. Thiomuscimol, dihydromuscimol, and gaboxadol [4,5,6,7-tetrahydroisozazolo[5,4-c]pyridin-3-ol (THIP)] are all structural analogs of muscimol [65]. While some of these agents have better bioavailability than muscimol (gaboxadol crosses the blood-brain barrier more easily) [138], all cause side effects such as dizziness, vomiting, and strong sedation that precluded its use in treating pain, anxiety, epilepsy, etc [139].

Recently, it has been suggested that direct acting GABAmimetic drugs, especially gaboxadol, could potentially treat sleep disorders [65, 139]. GABAergic compounds acting at benzodiazepine sites such as zolpidem reduce latency to sleep, and are widely prescribed for the treatment of insomnia [140]. However, several side effects of these drugs include tolerance and dependence with long term use as well as "grogginess" and hang-over effects possibly due to disruptions in slow-wave sleep [65, 140]. Drugs like gaboxadol and muscimol may not only shorten the onset of sleep, but also prolong the duration of slow-wave sleep thereby increasing the duration and quality of sleep [141-143]. Differences between the two classes of sleep drugs may lie in their mechanisms of action. While both benzodiazepine site ligands and direct acting

$GABA_A$-R agonists are GABAergic, the two classes of drugs likely act on different targets. Benzodiazepine site ligands produce their hypnotic effects through synaptic α1βγ2 receptors [134, 136] whereas gaboxadol is likely to act through extrasynaptic receptors (e.g. those containing the δ subunit).

1.6.3.1 Extrasynaptic receptors and direct $GABA_A$-R agonists

Extrasynaptic $GABA_A$ receptors mediate tonic inhibition, which is the continuous activation of receptors located outside the synaptic cleft by low steady-state concentrations of GABA. Examples of extrasynaptic receptor subtypes include α4βδ in thalamic relay neurons [111] and dentate granule cells [109], α5β3γ2 in hippocampal CA1 pyramidal cells [97] and α6βδ in cerebellar granule cells [57].In recombinant receptors, GABA has a greater affinity for receptors containing α6 and α5 than those containing α1, α2 and α3 [144]. Using electrophysiological methods, recombinant receptor subtypes found extrasynaptically have greater GABA sensitivity relative to synaptic $GABA_A$-Rs; α6β3δ receptors have a 5-10 fold lower EC50 than α6β3γ2 receptors [117, 118] and a forty-fold lower EC50 than α1β2γ2 receptors [118]. Similarly, GABA was 5-fold more potent on α4β3δ than α4β3γ2 receptors [116].

Extrasynaptic $GABA_A$-Rs are also more sensitive to the direct GABAmimetic agents, gaboxadol and muscimol compared to synaptic receptors. Both muscimol and gaboxadol are 10 fold more potent for α6β3δ than α6β3γ2 receptors [72, 144] and gaboxadol is 15 fold more potent on α4β3δ compared to α4β3γ2 [116]. Perhaps the most interesting pharmacological finding with muscimol and gaboxadol is their efficacies on various receptor subtypes. Compared to potentiation by GABA, muscimol is 40% and 80% more efficacious toward α4β3δ and

α6β3δ, respectively, while gaboxadol is two-fold and three-fold more efficacious on extrasynaptic receptors [116, 144]. Therefore, muscimol and gaboxadol are "super-agonists" toward δ containing receptors, and GABA, the endogenous ligand is a partial agonist on these receptors. In contrast, gaboxadol is 25% and 50% less efficacious than GABA on α1β2γ2 and α3β3γ2 receptors respectively indicating that gaboxadol is only a partial agonist on the majority of synaptic $GABA_A$-Rs [144]. Furthermore, binding studies using [^{3}H]-muscimol demonstrated that recombinant receptors containing the δ subunit had 5-fold higher affinity [145] compared to those containing γ2 and that mice lacking $GABA_A$-R α6 [146] and δ [147] subunits have reduced muscimol binding using *in situ* autoradiography.

1.6.4 Extrasynaptic $GABA_A$ receptors in the pharmacology of ethanol

The effects of ethanol on human behavior are well documented [148]. At low blood ethanol concentrations (< 25 mM), there is a feeling of euphoria, disinhibition, and decreased anxiety. As the concentration of ethanol in the blood increases, motor function is impaired and speech becomes slurred. With blood alcohol concentrations greater than 45 mM, vomiting can occur and the subject can fall into a stupor. Blood concentrations higher than 100 mM can produce respiratory failure and death. Note that, although ethanol has dramatic effects on the CNS, ethanol is not a potent drug. Ethanol produces behavioral effects at low millimolar concentrations- in fact, the legal driving limit in most states is 0.08 % or approximately 17.4 mM. Ethanol's CNS depressant effects are similar to behavioral effects produced by other $GABA_A$-R agonists like benzodiazepines and barbiturates.

Many studies have implicated $GABA_A$-Rs in the mechanism of action of ethanol at the cellular level (for review see [149]). Pharmacologically relevant concentrations of ethanol can potentiate GABA activated ion current in isolated neurons [150-152]. On the other hand, other studies have found that ethanol inhibits [153] or does not affect [154, 155] these currents. One explanation for this inconsistency is that like benzodiazepines (see Section 1.6.2), ethanol may only potentiate $GABA_A$-Rs of a particular subunit composition. Therefore, extensive investigation of the subunit requirements has been undertaken, mostly via heterologous expression systems.

Many studies have found that that a variety of $GABA_A$-R subunit combinations, are sensitive to ethanol [73, 74, 156-161]. Interestingly, the majority of these studies have found that the concentration required to potentiate these receptors is often greater than 60mM, a concentration much higher than those achieved during social drinking. If $GABA_A$-Rs are essential for mediating the behavioral effects of ethanol, one might expect that they would respond to much lower concentrations that cause mild behavioral effects (for example, the legal limit for driving in many states 0.08% or ~17 mM). Recently, some studies have indeed suggested that there may be a subpopulation of $GABA_A$-Rs that are sensitive to low concentrations of ethanol. $GABA_A$-Rs found extrasynaptically (those containing the δ subunit) were potentiated by concentrations of ethanol that approximate those achieved by social drinking (<30 mM) [73, 159, 162, 163]. Recombinant $GABA_A$-Rs containing either α4 or α6 and δ were potentiated by 1-3 mM ethanol, a concentration achieved by less than half a glass of wine [73, 159]. In contrast, synaptic $GABA_A$-Rs (those containing γ2) were potentiated by much higher concentrations of ethanol [73, 159] which were in accord with other studies [156-158]. Additionally, the ethanol behavioral antagonist Ro15-4513 was found to inhibit ethanol action at

α4/δ receptors [164] and ethanol was found to block binding of tritiated Ro15-4513 to α4β3δ receptors [165]. It is important to note, however, that other laboratories did not corroborate these findings [74, 166]). Borghese et al. reported that neither α4/δ nor α6/δ receptors were more sensitive to ethanol than γ2-containing receptors [74]. Also, Yamashita et al. reported that ethanol had either no effect, or an inhibitory effect on GABA gated current of $GABA_A$-Rs containing α4/δ or α6/δ [166].

Experiments utilizing rats with a naturally occurring allelic variation of the α6 subunit also suggest that extrasynaptic $GABA_A$-Rs mediate behavioral effects of ethanol. Rats with the Q100 allelic variation of the α6 subunit of the $GABA_A$-Rs were more sensitive to the motor-incoordinating effects of ethanol [162]. The same study found that 1) potentiation of tonic current by ethanol was significantly greater in the α6 expressing cerebellar granule cells of α6(Q100) rats than α6(R100) rats and 2) ethanol more robustly potentiated in vitro receptors containing α6(Q100)β3δ than α6(R100)β3δ [162]. These findings, however, were directly contradicted by another laboratory [167, 168]. The laboratory of C.F. Valenzuela found no difference in the ethanol sensitivity of recombinant α6(Q100)β3δ compared to α6(R100)β3δ [168] and no difference in ethanol potentiation of GABAergic currents in (Q100) rats versus (R100) rats [167].

Other behavioral studies linking extrasynaptic $GABA_A$-Rs to ethanol action are evident in other rodent models. Progesterone-withdrawn rats that exhibit increased α4/δ expression were more sensitive to low concentrations of ethanol both at the cellular and the behavioral levels [159]. Finally, mice lacking the δ subunit of the $GABA_A$-R had reduced ethanol potentiation of tonic inhibition [163] and were found to have multiple defects with respect to alcohol-related behaviors. δ knockouts consumed less alcohol, had reduced convulsions following ethanol

withdrawal, and were less sensitive to the seizure-protecting effects of ethanol [169].

1.7 ANIMAL MODELS TO STUDY $GABA_A$-R PHARMACOLOGY

Recombinantly expressed $GABA_A$-Rs have served to identify structure- function relationships, but do not provide a model truly resembling that found *in vivo*. The exact relationship between subunit composition and receptor function has yet to be established due to the inability of many *in vitro* and *in vivo* studies to reach the same conclusion. For example, several *in vitro* studies suggested that inclusion of the long splice variant of the γ2 subunit conferred ethanol sensitivity to $GABA_A$-Rs [160, 161, 170] though this finding was not observed in other studies [155]. However, studies in γ2L knockout mice found no selective role of γ2L subunits in the acute behavioral effects of ethanol [171]. Conversely, in the late 1990s, a study using recombinant receptors reported no evidence for a role of the δ subunit in neurosteroid sensitivity [172], but δ subunit knockout mice exhibited a significant reduction in neurosteroid sensitivity [147]. However, it should be noted that subsequent *in vitro* studies have found that $GABA_A$-Rs containing δ subunits are potentiated by neurosteroids [116, 173][174].

These contradictory results demonstrate 1) the importance of conducting both *in vitro* and *in vivo* studies and 2) that attention must be given to the limitations of each method. Studies *in vitro* occur in non-neuronal cell lines in which an isolated receptor subtype is examined outside of its natural environment, however, the primary benefit of studying *in vitro* receptors is that it allows for the rapid study of several user-defined receptor subtypes. In contrast, *in vivo* studies are limited to the study of a diverse population of receptor subtypes making it difficult to

correlate function and receptor subunit composition. This problem is partially circumvented by using $GABA_A$-R subunit gene knockout animals. While production, care and expense of making such mouse strains presents a significant barrier, studies using these reagents are clearly essential to understanding, within the context of the whole animal, the contributions of particular receptor subunits in the mechanism of action of ethanol.

1.8 THE α4 SUBUNIT OF THE $GABA_A$-R

$GABA_A$-Rs containing the α4 subunit are highly expressed in the thalamus and dentate gyrus, with lower expression levels in cortex, striatum and other brain areas [53, 66, 175] . $GABA_A$-Rs containing α4 subunits are often found with the δ subunit extrasynaptically and are proposed to play a role in tonic inhibition [110, 111, 176-178], which is the continuous activation of receptors located outside the synaptic cleft by low concentrations of GABA. *In vitro*, α4 subunit-containing receptors are insensitive to diazepam, but show high sensitivity to other sedative-hypnotic drugs, including ethanol [73, 163], neurosteroids [109, 116, 173], etomidate [179], muscimol [72] and the novel hypnotic drug, gaboxadol (formerly known as THIP) [111, 116, 180].

In addition to the unique pharmacology of α4-containing $GABA_A$-Rs, there is substantial plasticity in the expression of the $GABA_A$-R α4 subunit. This phenomenon has been observed in a variety of experimental and pathophysiological conditions related to behavioral hyperexcitability. For example, α4 expression is markedly elevated following electroshock [181], alcohol exposure/withdrawal [60, 61, 178, 182], steroid withdrawal [183, 184], social

isolation [185], and epilepsy [62, 63]. The magnitude of alterations to α4 subunit levels is remarkable relative to those of other $GABA_A$-R subunits [60].

1.9 GAPS IN OUR KNOWLEDGE ABOUT α4

α4 containing $GABA_A$-Rs have interesting properties that differ from other subtypes. However, a direct cause and effect relationship between receptor structure and function has not been established. For example, while α4 subtypes are implicated in mediating tonic inhibition in the thalamus and dentate gyrus, this has not been determined unequivocally. In a similar vein, even though electrophysiological studies *in vitro* indicate that α4-containing $GABA_A$-Rs are highly sensitive to ethanol [73, 163], neurosteroids [109, 116, 173], etomidate [179] and gaboxadol [111, 116, 180], it is unclear whether these receptors mediate the behavioral effects of these drugs. Also, it is not clear, whether the plasticity of α4 expression is a cause of, or a consequence of, the neuronal hyperexcitability associated with each model syndrome. However, important clues suggesting the former have been provided using antisense α4 mRNA [183]. One approach to answer these questions would be use a selective inhibitor of α4 containing $GABA_A$-Rs, but unfortunately, such an agent is not currently available.

An alternative approach is to use genetic engineering technology. Genetically engineered animals are defined as those animals that have had their genome altered as a result of direct, investigator intervention. The two most widely used types of genetically modified animals are transgenic animals and gene-targeted mice, the latter including knockout and knockin mice. Transgenic animals are those genetically engineered animals that have an additional gene randomly added to their genome. Gene-targeted mice are those genetically engineered animals

in which an endogenous gene has been precisely modified. Gene targeted animals are created by homologous recombination in embryonic stem cell lines that are subsequently introduced into developing embryos. Knockout (or null) mice are the most commonly used gene-targeted animals. As the name suggests, knockout mice are genetically mutated such that a single gene can no longer produce functional protein. Gene knockout mice are used as reagents to discover or characterize the role of a single gene within a complex physiological context. For example, nullification of the α4 gene in a mouse could provide an effective model to understand the role of α4 in mediating the behavioral effects of alcohol and other sedative-hypnotic drugs. One could hypothesize that if a behavioral response to these drugs is mediated by α4-containing $GABA_A$-Rs, it would be diminished/ablated in α4 KO mice. However, α4 knockout mice are not available; therefore, one of the primary undertakings of this dissertation was to create and characterize such a strain (Chapter 3).

1.10 CREATING GENE KNOCKOUT MICE

Creating knockout mice is a lengthy and technically demanding process. The simplest approach to making a global knockout mouse is to replace a portion of an exon with a selectable marker (e.g. neomycin resistance) that will disrupt the coding sequence of the gene of interest (see **Fig. 1A**). Because the process of creating a global gene knockout has many steps and is time consuming, it is important to consider some of the pitfalls and limitations of such models before starting. For example, knockout of a particular gene can be neonatally or perinatally lethal. Unfortunately, mice that do not survive are not useful for whole animal behavioral studies (that are most interesting in studying α4). A second limitation is often observed in knockouts that do

survive: compensatory changes of other genes. For example, it has been found that knockout of specific $GABA_A$-R subunits can result in changes in expression of other subunits [186-190]. Furthermore, compensation can also occur in completely unrelated genes. For example, knockout of the α6 subunit of the $GABA_A$-R resulted in the upregulation of the TASK-1 potassium channel [122]. Such compensatory changes may confound the interpretation of resultant phenotypes. Finally, because global knockouts eliminate expression of the gene from every cell in the organism, it can be nearly impossible to determine which cell types are responsible for any observed phenotype.

To overcome many of the limitations of global knockouts, techniques have been developed for knocking genes out in specific tissues and/or at specific times in development. "Conditional knockout" technology relies on the use of gene targeting in embryonic stem cells and also relies on CRE-loxP system. CRE-loxP technology is a site-specific recombination system that was initially isolated from bacteriophage P1 [191]. LoxP sites are small (34 bp) sequences of DNA. CRE is an enzyme that catalyzes recombination between loxP sites. To use this system for creating conditional gene knockouts, one first has to genetically modify the gene of interest as illustrated in (**Fig. 1B**). Here, loxP sites flank an important exon of the gene of interest. Such a locus is termed a "floxed" locus. Because the loxP sites by themselves are generally innocuous when placed into intronic DNA, they should have no adverse effect on the gene of interest. A floxed gene will continue to be expressed and function normally. However, if CRE is introduced into a cell that has a floxed locus, the recombinase will recognize the loxP sites and induce site-specific recombination. CRE is routinely introduced by crossing a floxed mouse to a transgenic mouse that expresses CRE in a tissue specific pattern. The net result of CRE recombination is deletion of the intervening sequence, thereby inactivating the gene of

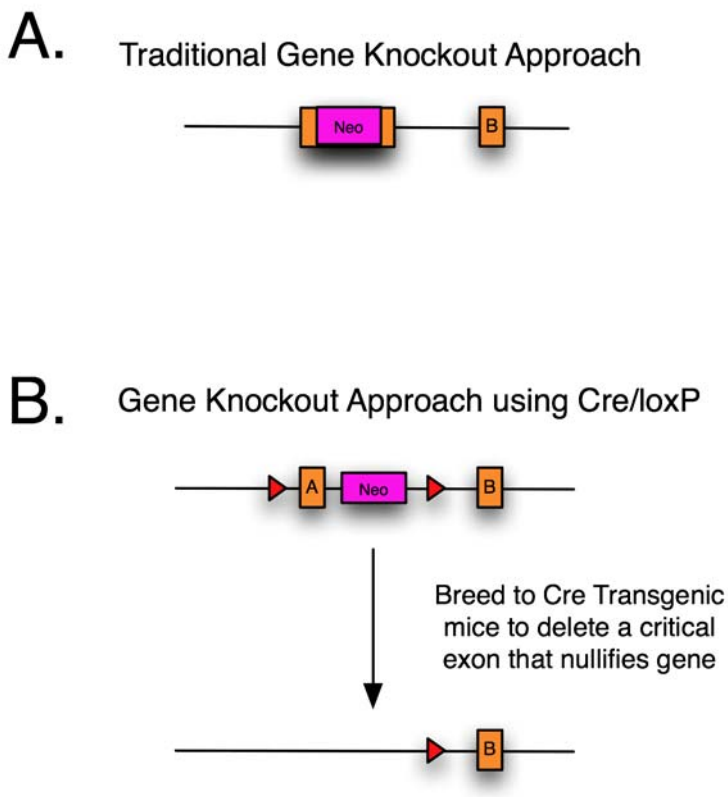


Figure 1. Traditional gene knockout approach versus conditional approach using Cre/Lox system. With the traditional approach, (A), a portion of coding exon is replaced with selection cassette to create a null allele. In contrast, using Cre/lox, (B), a critical exon is flanked by loxP sites. Subsequent introduction of Cre recombinase deletes DNA in between loxP sites and creates null allele. Cre recombinase can be introduced in all cells to create a global knockout or in some cells to create a conditional knockout. Exons (numbered orange boxes) , loxP sites (red triangles).

interest. By controlling which cells in the animal express CRE, one can knockout the gene of interest only in those cells thereby creating a conditional knockout. In cells that do not express CRE, the floxed gene of interest will continue to function normally. Alternatively, global knockout mice can be created from a floxed allele by mating the floxed mouse to a global deleter Cre transgenic mouse.

Conditional knockout mice circumvent many of the problems associated with global knockouts. For example, if compensation occurs during development or a gene is neonatally

lethal when globally knocked out, then a conditional knockout mouse line may be used to produce a postnatal knockout. The second major advantage of conditional knockouts is that by knocking out the gene of interest in a restricted cell population, one is able to assign a phenotype to a particular cell type. The only major downside to creating conditional knockout mice is that they are even more technically complex to construct and produce.

2.0 SPECIFIC AIMS

Despite ethanol's wide use over the duration of human history, the precise molecular targets through which ethanol produces its intoxicating effects are not well understood. Modulation of many different brain proteins by ethanol is likely to cause the altered behaviors [5]. One possible target is the primary inhibitory neurotransmitter receptor in the mammalian brain, the $GABA_A$ receptor.

$GABA_A$-Rs containing the α4 subunit are highly expressed in the thalamus and dentate gyrus, with lower expression in cortex, striatum and other brain areas [53, 66, 175]. $GABA_A$-Rs containing α4 subunits are often co-localized with the δ subunit extrasynaptically and are though to play a role in mediating tonic inhibition in the forebrain [110, 111, 176-178]. Studies using recombinant receptors show that α4 subunit-containing receptors have an interesting pharmacological profile with respect to GABAergic agents: these subtypes are insensitive to benzodiazepines, but highly sensitivite to ethanol [73, 163], muscimol (Ebert 2006) and the novel hypnotic drug, gaboxadol [111, 116, 180]. In light of these in vitro studies, *the central hypothesis* of this dissertation is that α4 *subunit-containing $GABA_A$-Rs mediate the behavioral effects of ethanol, gaboxadol and muscimol*. The use of gene knockout mouse models allows this hypothesis to be tested at the level of the whole organism. Specifically, the aims are to:

1. **Genetically engineer mice that lack the α4 subunit of the GABA-A receptor (Chapter 3)**. To create these mice, animals with a floxed α4 gene (i.e. a critical exon flanked by loxP sites) will be created. Subsequently, the floxed α4 mouse will be mated to a general deleter, Cre recombinase expressing strain to create animals that globally lack α4. Inactivation of the *Gabra4* gene will be confirmed by RNA and protein analysis. In the event that global KO mice do not provide an effective model for testing in vivo contributions of α4 containing $GABA_A$-Rs (i.e., if α4 KO mice are neonatally lethal), conditional knockout mice can be created from mice with the α4 floxed allele.

2. **Analyze α4 KO and δ KO mice with a battery of behavioral tests to assess phenotypic alterations in behaviors induced by muscimol and gaboxadol (Chapter 4) as well as ethanol (Chapter 5)**

Included will be behavioral tests for ataxia, analgesia, anxiolysis, sedation, hypnosis, hypothermia, and metabolism and clearance.

3.0 CREATION OF GABAA-R α4 SUBUNIT MUTANT MICE

3.1 INTRODUCTION

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. Its primary target, GABA type A receptors ($GABA_A$-Rs), are pentameric complexes that function as ligand-gated chloride ion channels. There are a variety of subunit families that make up $GABA_A$-Rs; a total of nineteen distinct subunits have been cloned, α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3 [43]. This diversity in subunit composition results in substantial anatomical, functional and pharmacological heterogeneity.

$GABA_A$-Rs containing the α4 subunit are highly expressed in the thalamus and dentate gyrus, with lower levels in cortex, striatum and other brain areas [53, 66, 175] . $GABA_A$-Rs containing α4 subunits are often found with the δ subunit outside of synapses and are proposed to play a role in tonic inhibition [110, 111, 176-178] which is the continuous activation of receptors located outside the synaptic cleft by low concentrations of ambient GABA. In vitro, α4 subunit-containing receptors are insensitive to diazepam, but show high sensitivity to other sedative-hypnotic drugs, including ethanol [73, 192], neurosteroids [109, 116, 173], etomidate [179] as well as the GABAmimetic drugs muscimol [72] and the novel hypnotic drug, gaboxadol (formerly known as THIP) [111, 116, 180]. In addition to the unique pharmacology of α4-containing $GABA_A$-Rs, there is substantial plasticity in the expression of the $GABA_A$-R α4

subunit that has been observed in a variety of experimental and pathophysiological conditions related to behavioral [60-63, 178, 181-185].

Clearly, α4 containing $GABA_A$-R subtypes have interesting properties that differ from other subtypes. The purpose of the following experiments is to create a model by which the contribution of α4 containing $GABA_A$-R subtypes can be assessed in the context of the intact organism. Mice that lack α4 will be genetically engineered. Despite the limitations of global knockout mice (See section 1.10), a global α4 knockout mouse strain will be invaluable in answering many scientific questions (and will be extensively used in the experiments in Chapter 4 and 5). However, it will be wise to create mice that provide the maximum versatility. Therefore, Cre/Lox technology will be used to create a mouse line with a "floxed" α4 locus. Floxed α4 mice will allow creation of not only a global α4 knockout but also, conditional α4 knockout mice, if needed.

3.2 METHODS

3.2.1 Overview of creating *Gabra4* mutant mice

A gene targeting construct was designed such that loxP sites flanked the third exon of the *Gabra4* gene (**Fig. 2**). Theoretically, deletion of this 68 base pair exon would introduce a frameshift mutation in the transcribed mRNA ultimately leading to a truncated peptide. A thymidine kinase (TK) cassette was inserted to produce negative selection and a neomycin resistance cassette (NEO) flanked by frt sites was inserted to produce positive selection. Due to cryptic splice sequences, the NEO cassette can impair normal gene

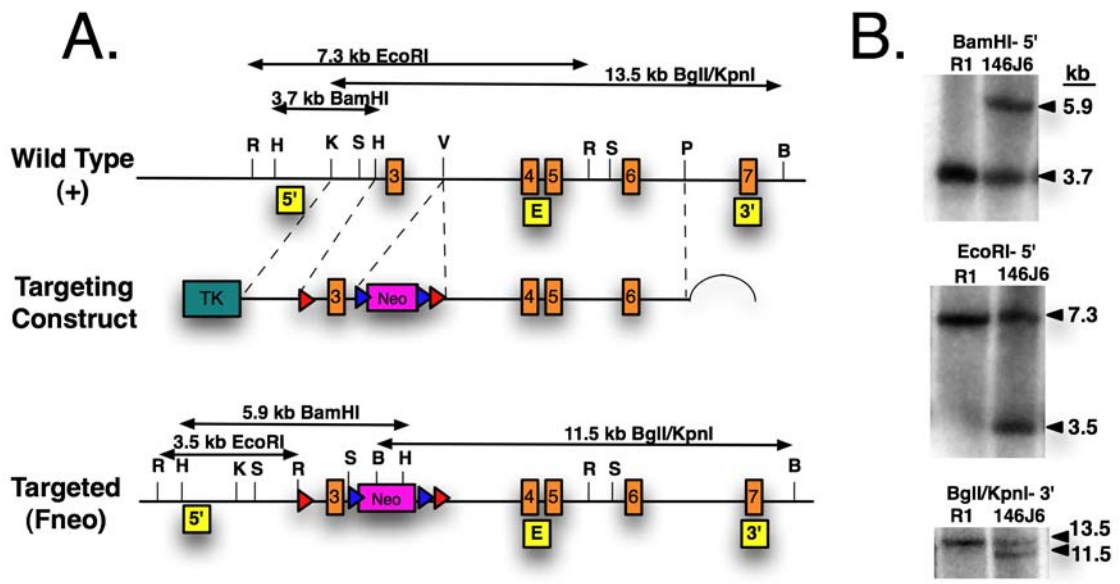


Figure 2. Targeted disruption of the *Gabra4* gene. *(A)* Targeting strategy used to produce α4 KO mice. Relevant region of endogenous wild type gene (+), targeting vector, and correctly targeted allele (Fneo). Relative locations of relevant restriction sites, B-BglI, R-EcoRI, K-KpnI, H-BamHI, V-EcoRV, P-SpeI, exons (numbered orange boxes), loxP sites (red triangles), frt sites (blue triangles), plasmid backbone (wavy line), probes (yellow boxes), and positive (NEO) and negative (TK) selection cassettes are shown. *(B)* Southern Blot analysis of parental R1 embryonic stem cells and 146J6 correctly targeted ES cells. For the 146J6 cells, the 5' probe hybridized to a 3.7 kb (endogenous) and 5.9 kB (targeted) BamHI digested fragment and a 7.3 kB (endogenous) and 3.5 kB (targeted) EcoRI fragment. The 3' probe hybridized to a 13.5 kB (endogenous) and 11.5 kB (targeted) BglI/KpnI doubly digested ES cell DNA.

transcription, even when inserted into intronic DNA [192-204]. Removal of NEO would be essential in creating a normally functioning floxed α4 allele for conditional knockout studies. This was accomplished using the FLPe/frt system [205, 206], a distinct site-specific recombinase system that is analogous to the Cre/lox system. FLPe deleted the NEO cassette flanked by frt sites while leaving exon 3 and the loxP sites intact.

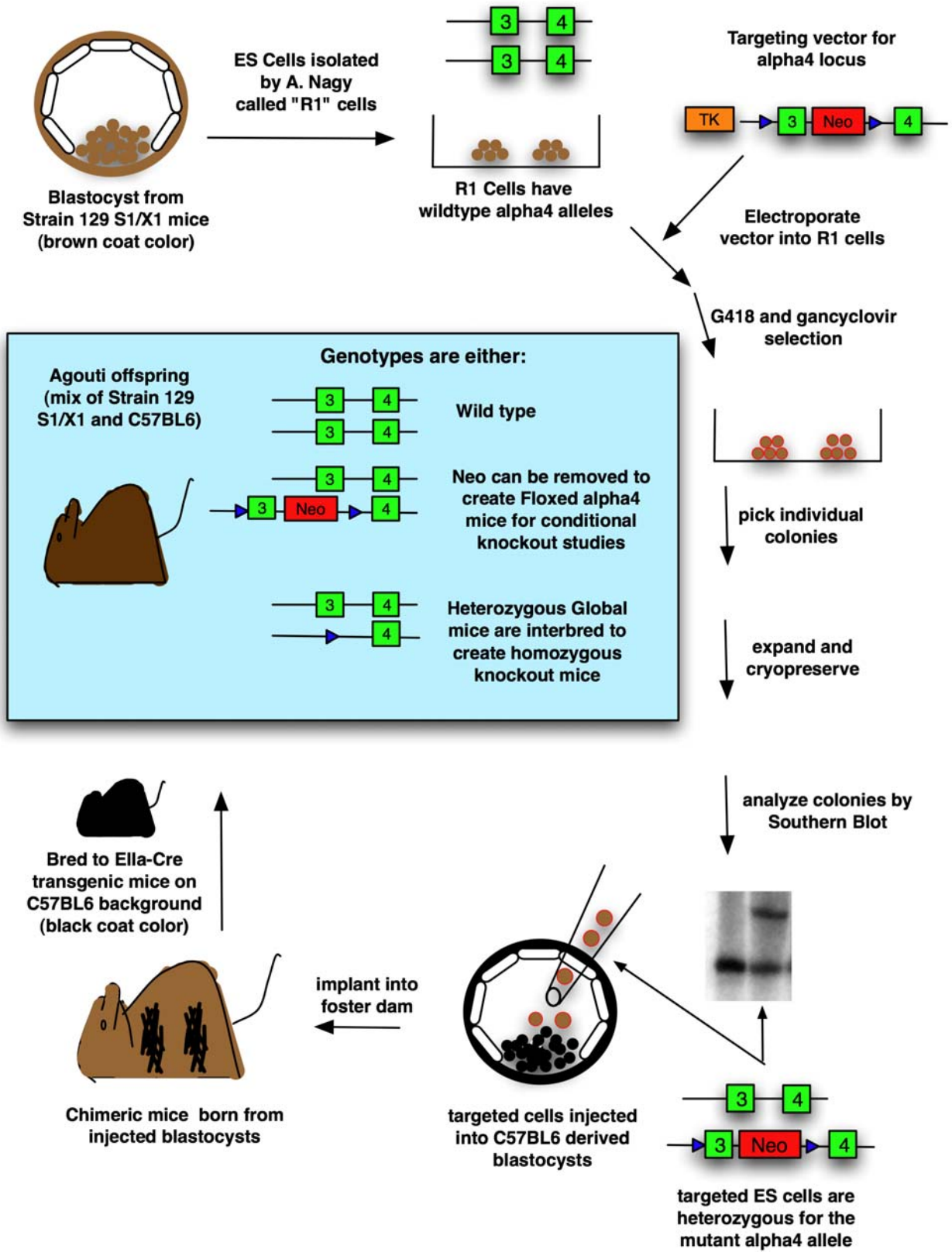


Figure 3. Schematic for creating gene targeted mice.

After creating the targeting vector, several steps were required to create gene targeted mice (**Fig. 3**). The targeting construct was electroporated into R1 cells [207]. This ES cell line is derived from a hybrid of two mouse strains: Strain 129 S1 and Strain 129 X1 mice. This cell line was chosen over cell lines from a pure Strain 129 X1 background [e.g. Go Germline ES Cells (Genome Systems, Inc., St. Louis, MO)] only because of the high success rate the Homanics Lab has in producing germ line competent chimeras from targeted R1 ES cells [171, 208-212]. Correctly targeted ES cells were then be microinjected into blastocysts (3.5 days post coitum) from C57BL/6J mice and the manipulated blastocysts were implanted into the uterus of pseudopregnant females. The mice that developed from the manipulated blastocysts were called chimeras because they possessed a mixture of two genetically distinct cell types; one derived from endogenous cells of the microinjected blastocyst, the other derived from the ES cells. Coat color was used to easily identify a chimera derived mostly from the targeted ES cells versus chimeras derived mostly from endogenous blastocyst ES cells. Chimeras had a mixture of black and brown coat color. Brown fur would be derived from injected, gene targeted ES cells (Strain 129) and black fur would be derived from endogenous ES cells of the injected blastocyst (C57BL/6J). Therefore, chimeras that were mostly brown are more likely to be derived from targeted ES cells. Because some of the gonadal tissue was derived from the altered ES cells, the chimeras were able to pass the targeted allele to its offspring. The targeted allele (Fneo) was converted to the global knockout allele (f) or to the floxed allele (F) that could later be used to generate conditional knockouts.

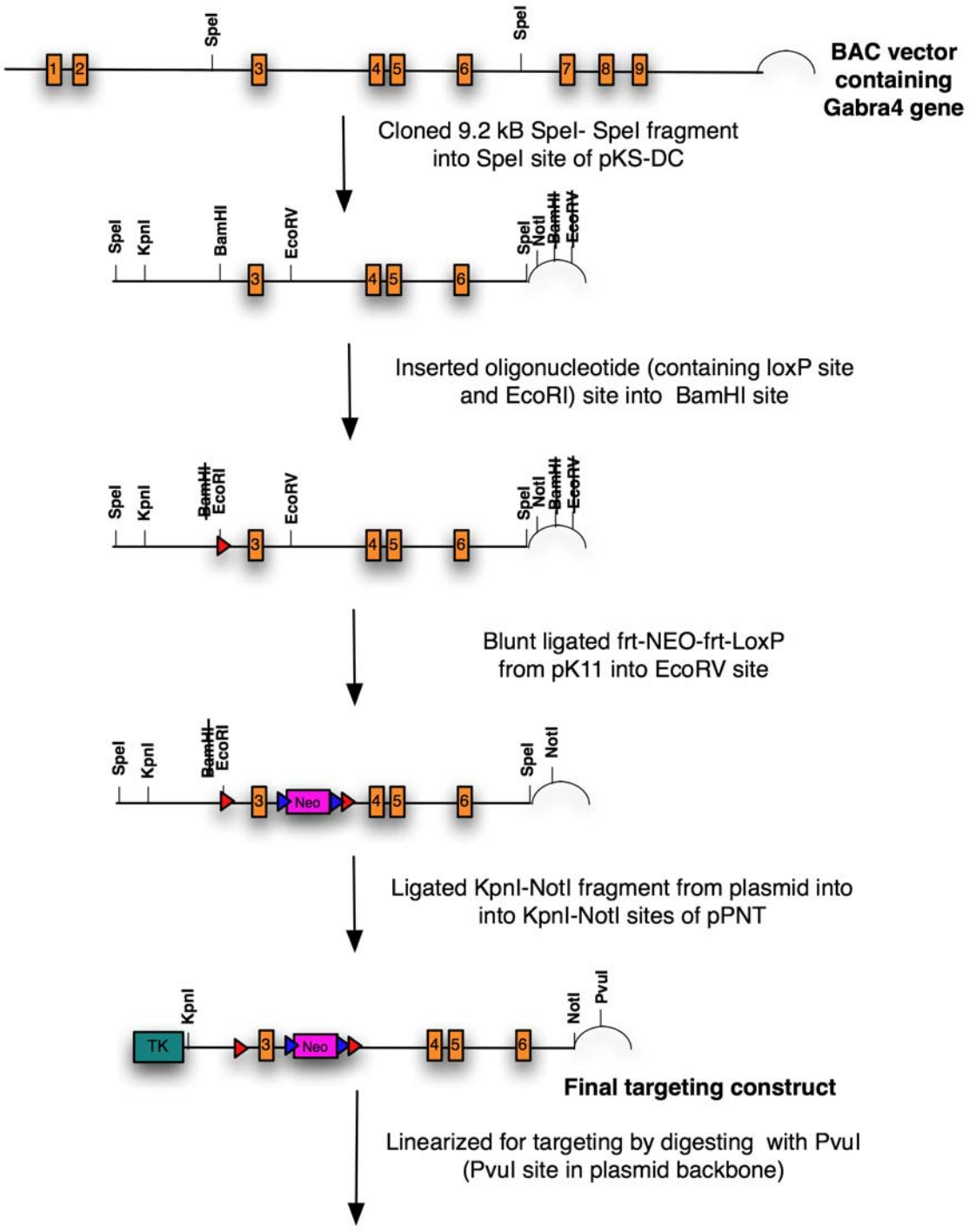


Figure 4. Strategy for creating gene targeting construct. Relevant restriction sites, exons (numbered orange boxes), loxP sites (red triangles), frt sites (blue triangles), plasmid backbone (wavy line), and positive (NEO) and negative (TK) selection cassettes are shown.

3.2.2 Design of α4 subunit gene targeting vector

The *Gabra4* gene targeting vector was created using standard recombinant DNA techniques (see **Fig. 4** for schematic). The targeting vector containing the *Gabra4* gene was constructed from a Strain 129 X1 mouse genomic DNA clone housed in a BAC vector (provided by Neil Harrison, Weill Medical College at Cornell University). Briefly, a 9.2 kB SpeI fragment containing Exons 3-6 was subcloned into *Bluescript KS* with destroyed BamHI and EcoRV sites. An oligonucleotide containing a loxP site and an EcoRI site was inserted into a BamHI site 625 base pairs 5' to Exon 3. This cloning step destroyed the BamHI site. A blunted fragment containing frt sites 5' and 3' of a neomycin resistance gene (NEO) and a loxP site 3' of NEO [204] was cloned into an EcoRV site 118 bp 3' to Exon 3. A NotI-KpnI fragment containing exons 3-6, loxP sites, NEO, etc. was then cloned into *pPNT* [213] such that a PGK driven thymidine kinase expression cassette was inserted 5' to α4 genomic DNA. The targeting construct was linearized with PvuI for targeting in ES cells.

3.2.3 Targeting of α4 in Embryonic Stem Cells

The linearized targeting construct was electroporated into R1 embryonic stem cells [207] following previously described procedures [209]. Briefly, G418 (270 ug/mL; Life Technologies, Gaithersburg, MD) and gancyclovir (2 μM; Sigma) resistant cells were screened for gene targeting by Southern blot analysis of BamHI digested genomic DNA using previously described procedures [209]. Fragments were hybridized with a 5' probe that was external to the targeting construct (**Fig. 2**). Proper targeting of the α4 locus was confirmed by Southern blot analysis of EcoRI digested DNA hybridized to the 5' probe and of BglI/KpnI digested digested DNA

hybridized to a 3' probe external to the targeting construct (**Fig. 2**). Please see Appendix A for information on how DNA probes were made.

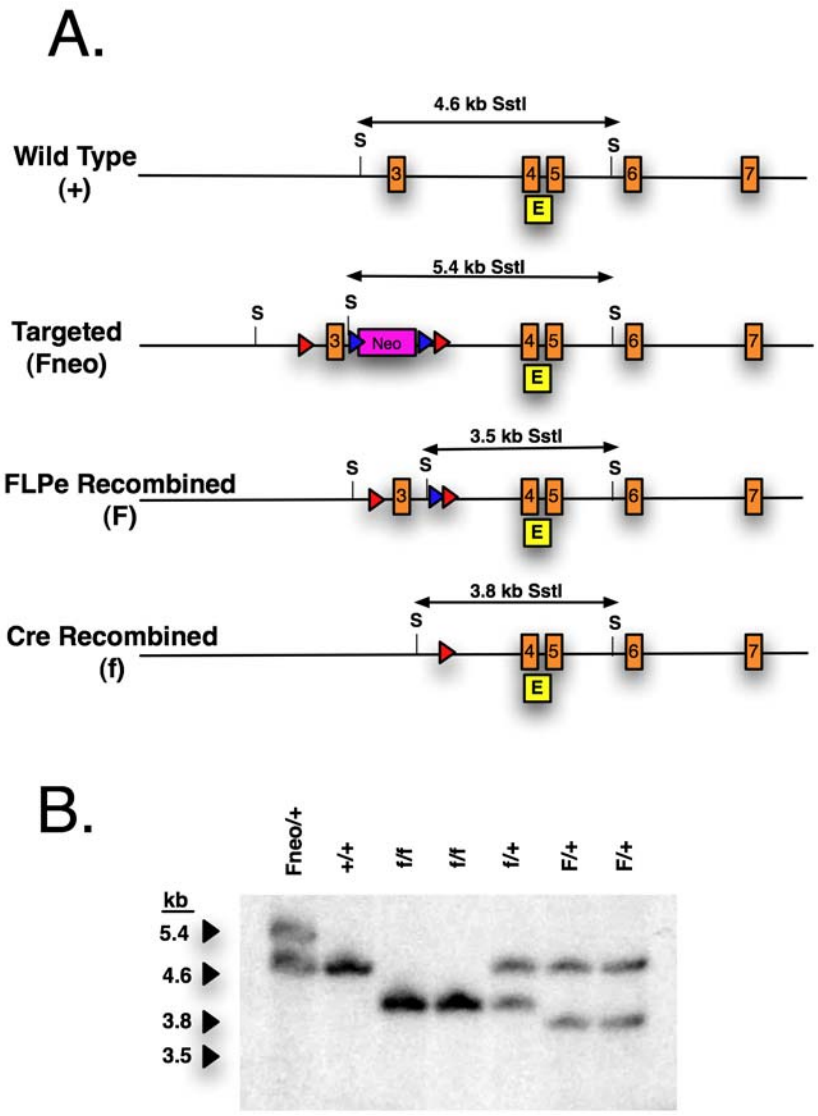


Figure 5. Gabra4 allelic variants created. ***(A)*** **Relevant region of endogenous wild type gene (+), correctly targeted allele (Fneo), FLPe recombined allele (F) and Cre-recombined (knockout) allele (f). S, Relative location of SstI restriction sites,** ***(B)*** **Southern Blot analysis of SstI digested tail DNAs. Probe E hybridized to a 4.6 kB (endogenous), 5.4 kB (targeted), 3.5 kB (FLPe recombined) and 3.8 kB (Cre-recombined) SstI digested fragment.**

3.2.4 Production of Mice from Targeted ES Cells

Three correctly targeted embryonic stem cell clones (145E1, 146J6 and 146L3) were microinjected into C57BL/6J blastocysts to produce chimeric mice. All of the results from the mice presented in this thesis are derived from the 146J6 ES cell clone. Chimeras were bred to EIIa-Cre transgenic mice (C57BL/6J genetic background) [214]. F1 agouti offspring were either wild-type (+/+), heterozygous for the targeted allele with NEO (+/Fneo) or heterozygous for the Cre-recombined (knockout) allele +/f (**Fig. 5**). Heterozygous knockout mice (+/f) were interbred to produce wild-type, heterozygous, or homozygous (f/f) knockout mice. +/Fneo mice were bred to actin-FLPe transgenic mice [215] (C57BL/6J genetic background) to remove NEO and create the floxed allele (F). Mice were genotyped using Southern blot analysis of SstI digested genomic DNA hybridized to a probe containing Exon 4 and 5 (**Fig. 5**). It should be noted that FLPe and Cre transgenes were "bred out" of the subsequent mouse lines. All mice were of F2-F6 generations and were of a Strain 129X1/S1 X C57BL/6J genetic background. All mice were housed under conditions of lights on at 7:00 and lights off at 19:00.

3.2.5 Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from hippocampus of WT and KO mice using TRIzol (Invitrogen, Carlsbad, CA). Approximately 200 ng of total RNA was used for RT-PCR (SuperScript One Step RT-PCR kit; Invitrogen) with primers specific for the *Gabra4* gene. Exon 2 (TCCCCAGGACAGAACTCAAAGG) and exon 5 (CATCCATAGGAAAATCCACCAGTC) primers correspond to nucleotides 224-245 and 674-651 of the α4 cDNA (GenBank accession number BC094603), respectively, and amplify a 453-bp fragment of WT mouse cDNA. RT-PCR

products were separated on a 1% agarose/TAE/EtBr gel and subsequently purified by using Zymoclean (Zymo Research, Orange, CA) and sequenced (DNA Sequencing Core Facilities, University of Pittsburgh, Pittsburgh, PA).

3.2.6 Western Blot Analysis

Following decapitation, thalamus, hippocampus and cerebellum were rapidly dissected, frozen on dry ice and stored at -70°C. P2 Membrane fractions were prepared by homogenization in PBS and subsequent centrifugation at 1,000g for 10 min followed by centrifugation of the supernatant at 10,000g for 25 min. Resultant pellets were resuspended and quantified using a bicinchonic acid method. Proteins (30 ug/lane) were electrophoresed on an SDS- 10% polyacrylamide gel. Proteins were transferred onto a poly-vinylidene difluoride membrane (BioRad, Hercules, CA). Blots were first probed with anti-$GABA_A$-R α4 subunit specific antibody [(1:1,000 dilution) raised against the C-terminus of the α4 protein ([78], Novus Biologicals, NB 300-193, Littleton, CO) and then with HRP-conjugated goat anti-rabbit secondary antibody (Novus Biologicals). Specific peptide labeling was detected by enhanced chemiluminescence (Western Lightning; Perkin Elmer, Boston, MA). Blots were later stripped using Re-blot (Chemicon International) and reprobed with β-actin polyclonal antibody (1: 10000 dilution, ab8227-50, Abcam) to verify equal loading of protein.

3.2.7 Elevated Plus-Maze Test

Basal anxiety-like behavior was tested using the elevated plus-maze. All mice were between 7 and 9 weeks of age and were tested between 13:00 and 16:00. Each mouse was placed on the

central platform of the maze, facing an open arm and allowed to freely explore the maze for 5 min under ambient room light. Open-arm and closed-arm entries and the cumulative time spent on the open and closed arms was recorded. A mouse was considered to be on the central platform or on an arm when all four paws were within its perimeter. The percent open-arm entries, total number of entries, and percent time in open-arms was determined. Data were analyzed using one-way ANOVA.

3.2.8 Open Field Activity

Basal motor activity of mice was determined using the open field assay. 8-10 week old mice were placed into a walled arena (43.2 cm x 43.2 cm x 30.5 cm) for 5 min. Distance traveled (cm) was measured automatically using an Activity Monitor (Med Associates, St. Albans, VT). All tests were performed between 12:00 and 16:00. Effect of genotype on basal motor activity was compared using Student's t-test.

3.3 RESULTS

3.3.1 Creation of Vector and ES Cell Targeting:

The DNA targeting vector was created as described (**Fig. 4**). Restriction digests and DNA sequencing confirmed the presence of all relevant restriction enzyme sites, the integrity of all exons, and the proper orientation of loxP and frt sites.

3.3.2 ES Cell Targeting

Over 1300 G418 and gancyclovir resistant colonies were isolated. Of these, 773 colonies were analyzed by Southern Blot. Six out of 773 ES cell clones displayed the predicted restriction fragment length polymorphisms indicative of the targeting event (**Fig. 2A***)*. Results from one clone (146J6) are depicted (**Fig. 2B***)*. In this analysis, the 5' probe hybridized to a 3.7-kb BamH1 and a 7.3-kb EcoRI-digested fragment from the WT α4 allele (+) and a 5.9-kb BamHI and a 3.5-kB EcoRI-digested fragment from the targeted, Neo containing, allele (Fneo). These results indicated correct targeting of the *Gabra4* gene and also presence of the 5' lox P site. The 3' probe hybridized to a 11.5 kb BglI/KpnI double digested fragment from the + allele and to a 13.5 kb fragment from the targeted allele.

3.3.3 Production of α4 mutant mice from targeted ES Cells

Two correctly targeted cell lines, 146J6 and 146L3, yielded germ-line competent chimeric males. Chimeras were bred to *EIIa*-Cre transgenic mice [214] to selectively delete the DNA sequences located between the loxP sites, including exon 3 of the *Gabra4* gene and the Neo cassette. From this breeding, two separate lines of mice were created 1) Mice that were +/f were interbred to produce +/+ (WT), +/f, and f/f (KO) mice and 2) +/Fneo mice were bred to *actin*-FLPe transgenic mice [215] (C57BL/6J genetic background) to remove NEO and create mice heterozygous for the floxed allele +/F. All mice were genotyped at weaning using Southern blot analysis (**Fig. 5B***)*. In this analysis, Probe E hybridized to a 4.6-kB SstI fragment from the + allele, a 5.4-kb SstI fragment from the Fneo allele, a 3.5-kB SstI fragment from the F allele, and a 3.8-kb SstI fragment from the f allele. Mice of all genotypes were viable and overtly

indistinguishable from each other.

3.3.4 Gross Characterization of f/f (KO) mice

Mice that were +/f were interbred to produce +/+ (WT), +/f, and f/f (KO) mice. Of the first 1512 offspring genotyped, 383 (25.3%) were +/+, 745 (49.3%) were +/f, and 384 (25.4%) were f/f. These values are in accord with the 1:2:1 genotype frequency as expected by Mendelian genetics ($\varphi^2 = 0.22$, $df = 2$, $p = 0.9$). When offspring are divided by genotype and gender, ratios are also in accord with expected Mendelian genetics ($\varphi^2 = 3.01$, $df = 5$, $p = 0.7$, See **Table 2**) Thus, homozygous KO mice have normal viability. KO mice are normal in size. Body weights (g ± SEM) of 7- to 8-week-old male mice were 24 ± 1 for WT ($n = 7$) and 24 ± 1 for KO ($n = 10$). Body weights of 7- to 8-week-old female mice were 23 ±2 for WT ($n = 6$) and 21 ± 1 for KO ($n = 6$). Brain weights also were similar between genotypes (525 ± 8 for WT, 527 ± 10 for KO; weight in mg ± SEM; $n = 3$ per genotype). Additionally, histological analysis of brain found no gross differences between genotypes (Carolyn Houser, University of California- Los Angeles).

Table 2. Distribution by gender and genotype from +/f by +/f mating

Gender	Genotype	#	Expected #	%	Expected %
F M	+/+ +/+	197 186	189 189	13.0 12.3	12.5 12.5
F M	+/f +/f	362 383	378 378	23.9 25.3	25.0 25.0
F M	f/f f/f	179 205	189 189	11.8 13.6	12.5 12.5
	Total	1512			

3.3.5 RNA Analysis

RT-PCR analysis using α4 specific primers was performed to characterize the gene transcripts derived from the mutant allele. The predicted 453-bp fragment was amplified from WT RNA by using primers specific for exon 2 and exon 5 (**Fig. 6B**). Sequencing of the PCR product confirmed the presence of exons 2-5 (data not shown). The predicted 453-bp fragment could not be amplified from KO RNA. Instead, ~375 nt and ~150 nt products were amplified. Sequencing of these products showed that the ~375 nt fragment was identical to the WT fragment except that nucleotides corresponding to exon 3 were absent. The ~150-nt fragment also was identical to wild type, except that nucleotides corresponding to exons 3 and 4 were absent (data not shown).

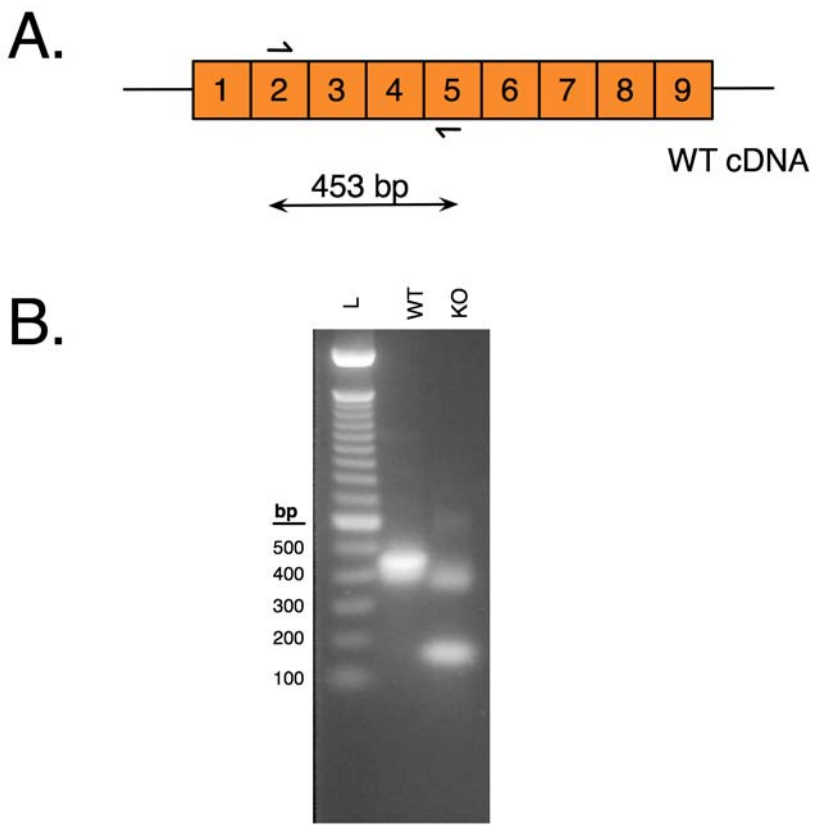


Figure 6. RNA analysis of α4 KO mice. (A) Schematic diagram of α4 cDNA. (B) RT-PCR products of WT (n = 1) and KO (n = 1) hippocampal RNA. WT cDNA amplifies a 453 bp fragment while KO cDNA amplifies a ~375 and ~150 bp fragment. Orange boxes represent exons. Arrows represent primer locations.

3.3.6 Protein Analysis

Western blot was used to examine whether deletion of exon 3 of the Gabra4 gene eliminated α4 protein. In membrane preparations of hippocampus and thalamus from WT mice, an α4-specific antibody [78] specifically recognized a ~67-kDa protein (Fig. 7). This protein band was absent in membranes from KO mice. As an additional negative control, protein from cerebellum, a brain region that lacks α4, was analyzed. As expected, α4 protein was absent in both WT and KO samples (Fig. 7).

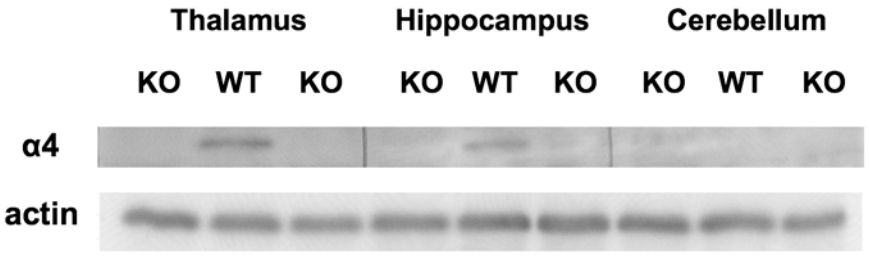


Figure 7. Western Blot analysis of thalamic, hippocampal and cerebellar membranes from α4 WT and KO mice. The ~67 kDa immunoreactive α4 protein present in hippocampal and thalamic WT samples is completely absent from KO samples, and as expected, is also absent from cerebellar preparations of WT and KO mice.

3.3.7 Behavioral Characterization

KO mice were overtly indistinguishable from WT mice. KO mice did not display any obvious behavioral abnormalities such as spontaneous seizures, hypoactivity, hyperactivity, or aggression toward cagemates.

Differences in anxiety and total activity of α4 KO mice were examined using the elevated plus maze. The elevated plus maze is widely used to assess anxiety-like behavior in response to the aversive stimulus of an elevated exposed space. On this test apparatus, knockout mice did not significantly differ from WT in open arm entries (**Fig. 8A**) or in time on open arms (**Fig. 8B**) suggesting that there is no change in anxiety-like behavior. KO mice had the same number of

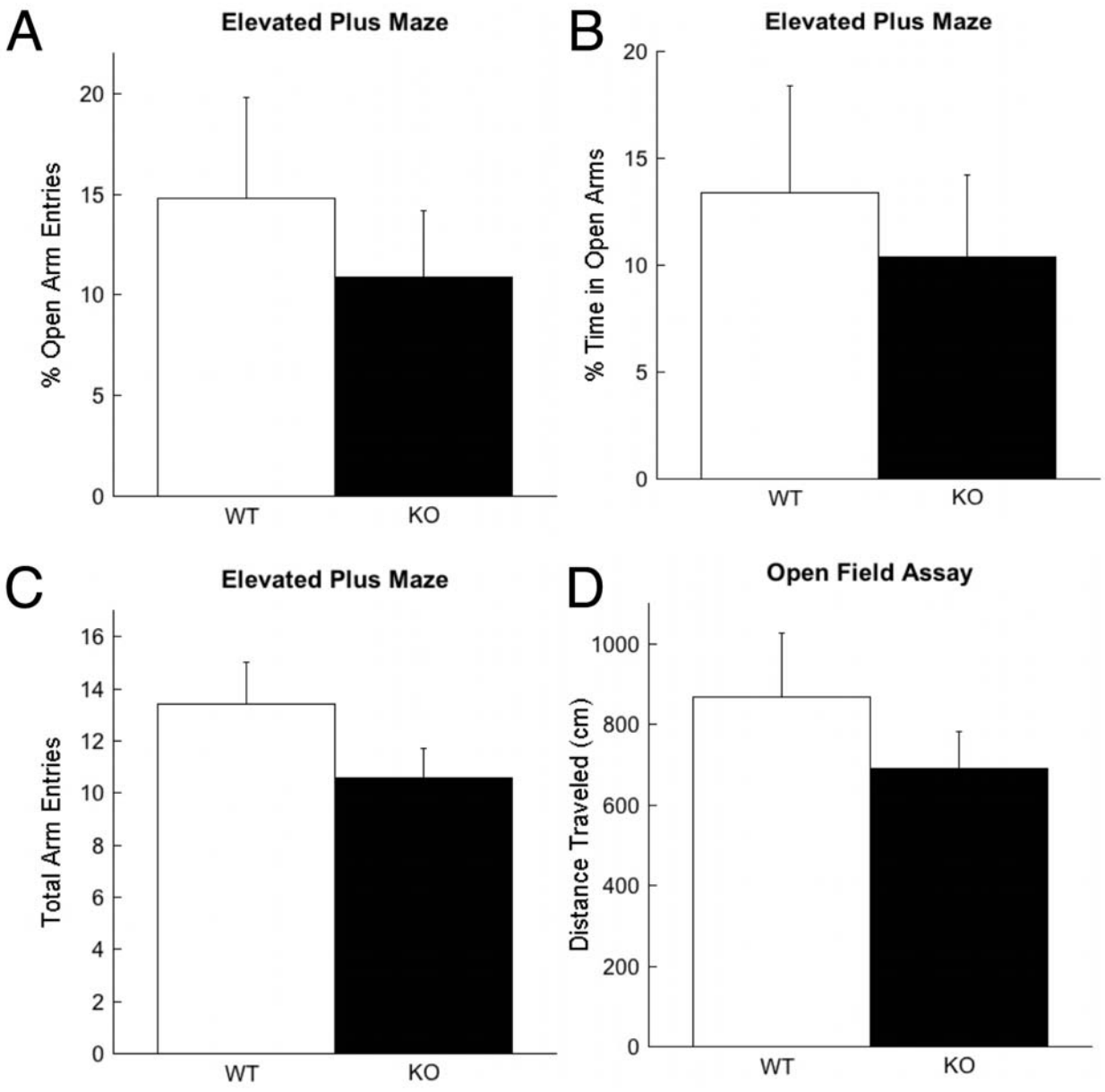


Figure 8. Behavioral characterization of α4 KO mice. (A-C) Elevated plus maze (n = 16 WT, 14 KO). α4 knockout mice did not differ from WT mice in (A) open arm entries, (B) time in open arms, or (C) in total number of entries. (D) Alpha4 KO (n = 14) mice were equally active in an open field as WT mice (n = 13).

total arm entries as wild types (**Fig. 8C**) reducing the likelihood that an alteration in locomotion masks a difference in anxiety-like behavioral measures on this assay.

KO mice were also tested for behavioral response to an aversive, brightly lit open field test apparatus using the open field assay. KO mice had equal locomotor activity compared to wild type mice when placed into a test arena (**Fig. 8D**) indicating that overall activity levels were similar for KO and WT animals.

3.4 SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

3.4.1 Summary

$GABA_A$-Rs containing the α4 subunit have attracted a great deal of attention despite their limited abundance in the brain, partly because of its 1) specific localization in the thalamus, dentate gyrus and striatum, 2) proposed role in mediating tonic inhibition, 3) unique *in vitro* pharmacological properties with respect to endogenous and exogenous ligands, and 4) plasticity of expression in a number of pathophysiological and experimental conditions. Mice with targeted mutations in the *Gabra4* gene were created to provide a model for understanding the physiological role of α4-containing $GABA_A$-Rs. α4 mutant mice were genetically engineered using Cre/lox, standard gene targeting and embryonic stem cell technologies. Both a global α4 KO strain and a floxed α4 strain were created, the latter for use in conditional α4 KO studies.

3.4.2 Creation and Characterization of α4 KO mice

Global α4 subunit knockout mice were homozygous for the Cre-recombined allele

lacking exon 3 of the *Gabra4* subunit gene (f/f). RT-PCR demonstrated that mRNA coded by exon 3 was eliminated in f/f (KO) mice thereby confirming that Gabra4 is a single copy gene. Western blot analysis of KO mice demonstrated that α4 protein truly is absent in this strain. Laboratories of Richard Olsen and of Carolyn Houser, both at the University of California, Los Angeles, confirmed elimination of α4 through western blot and immunohistochemistry, respectively (See Appendix B). Together, these results prove that deletion of exon 3 of the Gabra4 gene prevented production of α4 protein, i.e., resulted in a true knockout allele.

α4 KO mice were overtly normal and indistinguishable from wild-type littermates. KO mice had normal viability as well as similar body and brain weights as WT mice. This demonstrates that the Gabra4 gene is not essential for life even though it is expressed as early as E17 [216]. The normality of α4 KO mice is similar to that of other $GABA_A$ receptor subunit knockout models including α2 [217], α3 [218], α5 [219], α6 [220], β2 [221]and δ [147]. In contrast, mice lacking the β3 or γ2 subunits had severe developmental abnormalities. Most γ2 KO [222] or β3 KO [208] mice died within 48 hours of birth and over 90% did not live past 21 days of age. Those γ2 or β3 knockout mice that did survive had a much shorter life span and had multiple defects that were clearly detected by visual inspection such as hyperactive behavior, sensorimotor deficits and impaired coordination. Mice lacking the α1 subunit were impaired but less so. α1 KO mice had essential tremor [221, 223, 224] and less than 60% survived past 21 days [221]. The results from γ2 and β3 KO mice confirm that $GABA_A$-Rs are essential. The relative normality of α1 KO mice shows the extraordinary plasticity demonstrated by the CNS following deletion of a subunit that is incorporated into nearly half of all $GABA_A$-Rs. Similar compensatory plasticity in α4 KO mice may also account for the overt normality in this strain. Thus far, it has been found that there is an upregulation in γ2 in the dentate gyrus [225], as well

as an increase in α1 and α2 and a decrease in δ in the thalamus of α4 KO mice [226]. These compensatory changes do not make α4 KO mice useless, however, they are important to consider when drawing conclusions about the impact of deletion of the *Gabra4* gene.

Performance on the elevated plus maze and open field assays are used to assess more subtle changes in behavior. α4 KO mice performed similarly to WT in both assays indicating that KO mice had similar anxiety and locomotor activity levels. Other $GABA_A$-R subunit mutant strains have been tested using similar measures. Mice with reductions in γ2 had greatly reduced anxiety and reduced locomotor activity as measured by these same assays [212, 227]. α3 KO mice were more active in an open field [218] while α2 KO mice were less active [217]. Clearly, the presence or absence of certain $GABA_A$-R subtypes affect performance in these behavioral assays, however, the α4 containing $GABA_A$-R subtypes are not essential. A possible explanation for this is that the brain regions containing α4 (e.g., thalamus, striatum, hippocampus, etc.) are not important in these behaviors.

3.4.3 Future uses of α4 subunit KO mice

Qualitative behavioral assessment, elevated plus maze and open field data suggest that α4 KO mice behave normally. However, many subtle behaviors could be altered in α4 KO mice. For example, α4 is highly expressed in the thalamus, a brain region that relays sensory input to the cerebral cortex. Therefore, vision, hearing, taste or touch could be affected in α4 KO mice. The thalamus also plays a role in regulating sleep and wakefulness; suggesting these behaviors should also be examined. α4 is expressed in the dentate gyrus and CA1 region of the hippocampus, a region known to function in learning in memory. A number of rodent learning

and memory paradigms such as the Morris water maze, the Barnes maze, passive avoidance, fear conditioning and motor learning tasks could be used to assess learning and memory deficits (or enhancements) in α4 KO mice. Thus far, preliminary studies have found that α4 KO mice show enhanced trace and contextual fear conditioning [228]. Certainly, there is much more to be learned about the role of α4 containing $GABA_A$-Rs in learning and memory as well as behavior in general. $GABA_A$-R α4 KO mice will continue to be very useful in this regard.

There is substantial plasticity in the expression of the $GABA_A$-R α4 subunit which is especially intriguing because it manifests in a variety of experimental and pathophysiological situations leading to hyperexcitability. α4 expression is markedly increased by electroshock [181], alcohol exposure/withdrawal [60, 61, 178, 182], steroid withdrawal [183, 184], social isolation [185], and epilepsy [62, 63]. α4 KO mice could help understand whether there is a cause and effect relationship between increased α4 expression and behavioral hyperexcitability. For example, KO and WT littermate mice could be subjected to withdrawal from chronic alcohol administration and then examined for convulsions, a common effect of alcohol withdrawal. If α4 KO mice have fewer convulsions after alcohol withdrawal, this may suggest an important role for $GABA_A$-R α4 containing subtypes. Studies such as these are underway in the Homanics Lab.

Using *in vitro* expression systems, α4 subunit-containing $GABA_A$-R receptors have a unique pharmacology with respect to GABAergic ligands. α4 subtypes have high sensitivity to ethanol [73, 163], neurosteroids [109, 116, 173], etomidate [179] as well as GABAmimetic drugs such as muscimol [72] and gaboxadol [111, 116, 180]. This dissertation will use α4 subunit knockout mice to study the role of α4 containing $GABA_A$-R subtypes in mediating the behavioral effects of muscimol, gaboxadol (see Chapter 4) and ethanol (see Chapter 5). For these purposes, full viability and overt normality of α4 KO mice is very fortunate, unlike γ2 KO mice in which

neonatal lethality rendered them nearly useless for studying the mechanisms of ethanol and sedative-hypnotic drugs.

3.4.4 Conditional α4 Knockout mice

Another goal, in addition to creating global α4 KO mice, was to create mice with a floxed α4 locus (F) for conditional α4 knockout studies. The floxed locus created contained two loxP sites that flanked exon 3 and one frt site. It was predicted that the short length of these three recombination recognition sites, and their placement in intronic DNA, would allow the floxed gene to function normally. Normal gene expression in homozygous floxed (F/F) mice was studied by immunohistochemical analysis (Carolyn Houser, UCLA) where it was found that F/F mice had α4 subunit immunostaining patterns identical to wild-type mice, +/+ (data not shown). The floxed α4 mice created here add to the growing number of floxed $GABA_A$-R subunit alleles which already includes α1 [229], β3 [230] and γ2 [231].

In general, floxed mice can be used for a multitude of experiments. They can be mated to a transgenic mouse that expresses Cre recombinase in a tissue-specific or temporally regulated manner to make conditional knockouts. Floxed mice can also be mated to transgenic mice that express Cre in a ligand-inducible manner, therefore placing the timing of the knockout under investigator control. Finally, Cre recombinase packaged in a viral vector can be stereotactically injected to specific locations in the floxed mouse line to create models where the location and timing of the gene knockout is controlled [232].

Conditional knockouts using the floxed α4 allele can answer questions that global α4 KO mice cannot. For example, the α4 subunit is expressed in a number of tissues including the thalamus, hippocampus, striatum and cortex. Therefore, any phenotype observed in α4 global

knockouts cannot be ascribed to a particular region. In contrast, floxed α4 mice can be bred to *αCamKII*-promoter driven Cre transgenic mice [233] to create hippocampal-restricted knockouts. Floxed mice can also be bred to potassium channel, *Kv3.2*-promoter driven Cre mice [234] to create thalamus specific knockout of α4. Also, because α4 begins expression at stage E17 [216], there could be developmental compensations in global α4 KO mice that could mask the true contributions of α4. This problem could be circumvented by breeding floxed α4 mice to an inducible Cre transgenic line [235, 236] or locally injected with the Cre gene to control the precise timing of the knockout [232].

4.0 THE EXTRASYNAPTIC $GABA_A$-R IN THE MOLECULAR MECHANISMS OF GABOXADOL AND MUSCIMOL

4.1 INTRODUCTION

4.1.1 Tonic inhibition and α4 containing $GABA_A$-Rs

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. Its primary target, $GABA_A$-Rs, are pentameric complexes that function as ligand-gated chloride ion channels. Two types of inhibitory neurotransmission are mediated via $GABA_A$-Rs [85, 86]. Phasic inhibition results from the activation of receptors at the synapse by intermittent release of high concentrations of GABA from presynaptic terminals. Tonic inhibition, in contrast, is mediated by the continuous activation of receptors located outside the synaptic cleft by low concentrations of GABA.

Anatomical and pharmacological evidence has long suggested that $GABA_A$-Rs containing the α4 subunit play a role in tonic inhibition [110, 111, 176-178]. In thalamic relay neurons and dentate gyrus granule cells, α4 containing $GABA_A$-Rs co-localize extrasynaptically with the δ subunit [111, 176, 178], which has been found to play a critical role in mediating tonic inhibition [109]. Pharmacological properties of recombinantly expressed $GABA_A$-Rs containing α4/δ suggest an important role in tonic inhibition; these $GABA_A$-Rs have a higher

affinity for GABA [72, 116] and slower rates of desensitization [116] [85, 86], relative to the classical synaptic $GABA_A$-Rs.

Recently, tonic inhibition in α4 knockout mice was studied using slice electrophysiology [237]. Dentate gyrus granule cells of α4 KO mice were highly deficient in tonic inhibition where picrotoxin-sensitive tonic currents were reduced by about 80%. Even more striking, however, was a complete loss of tonic current in thalamic relay neurons of the ventrobasal complex in α4 KO mice (Appendix C). These studies clearly demonstrate that $GABA_A$-Rs containing α4 subunits mediate tonic inhibition in the thalamus and dentate gyrus. Results from α4 KO mice nearly mirror those observed in the dentate gyrus [109] and more recently, in thalamic relay neurons [238] in mice lacking the δ subunit. Taken together, these findings imply that extrasynaptic α4/δ containing $GABA_A$-Rs are functionally significant.

4.1.2 Gaboxadol and α4/δ $GABA_A$-R pharmacology

Extrasynaptic $GABA_A$-Rs containing α4/δ subunits are insensitive to benzodiazepines, but show high sensitivity to other sedative-hypnotic drugs, including ethanol [73, 163], neurosteroids [109, 116, 173], etomidate [179], muscimol [72] and gaboxadol (formerly known as THIP) [111, 116, 180]. Among these, gaboxadol recently gained much attention due to its promise as a novel hypnotic agent [65]. Gaboxadol is a direct $GABA_A$-R agonist as it acts at the same site as GABA. Like GABA, gaboxadol has a higher affinity for α4/δ than for synaptic $GABA_A$-Rs [116]. Gaboxadol also has two to three fold greater efficacy than GABA on α4/δ $GABA_A$-Rs essentially making it a "superagonist" for these receptors [72, 116]. In contrast, gaboxadol has either a partial or full agonist at the most common synaptic $GABA_A$-Rs, such as α1β2γ2, α2β3γ2 and α3β3γ2 [70, 144].

The preference of gaboxadol toward α4/δ containing $GABA_A$-Rs is illustrated in cellular studies. Thalamic relay neurons from the VB complex contain both α4/δ and α1/γ2 $GABA_A$-Rs [111]. Electrophysiological recordings from these cells found that low concentrations of gaboxadol, 100-300 nM, enhanced tonic inhibitory currents but had no effect on synaptic currents [111]. The importance of α4-containing $GABA_A$-Rs to gaboxadol action was demonstrated unequivocally using α4 KO mice ([237], Appendix D). Recordings from wild-type thalamic relay neurons showed that 100-300 nM gaboxadol elicited a reproducible and concentration-dependent current response. However, this response was completely absent in the neurons from α4 KO mice. It should be noted that higher concentrations of gaboxadol (1 μM) produced inward currents in both the WT and KO mice. Nevertheless, these results underline the selectivity of gaboxadol for $GABA_A$-Rs that contain the α4 and δ subunits over $GABA_A$-Rs that contain the α1 and γ2 subunits [111].

Gaboxadol has analgesic, sedative-hypnotic, anti-convulsive, anxiolytic and ataxic effects [138]. While selectivity of gaboxadol for α4/δ receptors has been clearly established at the molecular and cellular level, it is unknown whether these receptors mediate gaboxadol's behavioral effects. Synaptic $GABA_A$-Rs, such as α1β2γ2, are present in far greater numbers than extrasynaptic receptors [77, 78]. Therefore, synaptic $GABA_A$-Rs could mediate gaboxadol's behavioral effects even though they are less sensitive to the drug than extrasynaptic $GABA_A$-Rs. Still, the importance of α4/δ is highly likely; animals where extrasynaptic $GABA_A$-Rs are upregulated are more sensitive to the behavioral effects gaboxadol [239, 240].

4.1.3 Aims of this study

The primary purpose of the following experiments was to understand how gaboxadol achieves behavioral endpoints such as ataxia, sedation and analgesia. I hypothesized that the behavioral effects of gaboxadol would be diminished in mice lacking either α4 or δ subunits. In addition to gaboxadol, the behavioral effects of muscimol will also be tested. Muscimol is also a direct GABAmimetic drug that has been used extensively as a lead for the design of several other GABA analogs, including gaboxadol [138]. Similar to GABA and to gaboxadol, muscimol has a lower EC50 for extrasynaptic $GABA_A$-Rs than synaptic receptors [72]. Muscimol has a greater maximal effect on extrasynaptic receptors than GABA but much less so than gaboxadol [70, 72, 144]. Behaviorally, muscimol produces similar effects as gaboxadol [241] [242] [243, 244]. In light of some important similarities between gaboxadol and muscimol, I hypothesize that the behavioral effects of muscimol may also be diminished in α4 KO or δ KO but not α1 KO mice.

4.2 MATERIALS AND METHODS

4.2.1 Mice

Three different $GABA_A$-R subunit knockout mouse strains were used to examine the behavioral mechanism of action of gaboxadol and muscimol. α4 KO and WT littermate controls were created by interbreeding mice heterozygous for the KO allele. α4 KO mice were of a mixed genetic background C57BL/6J and Strain 129S1/X1, F2-F6 generations. δ KO/WT (mixed C57BL/6J and Strain 129S1/X1 genetic background from > F20 generations, [147]) and α1

KO/WT mice (mixed C57BL/6J, FvB, and Strain 129S1/X1 genetic background from > F15 generations [211]) were created in a similar fashion. At weaning, mice were genotyped using Southern Blot analysis of tail DNA as previously described for α4 KO[237], δ KO [147] and α1 [211] KO mice. Mice were group housed, given free access to standard rodent chow and water, and maintained on a 12 h alternating light/dark schedule with lights on at 07:00. For all experiments, both male and female mice were used. Gender differences were observed in some cases (noted below). Wherever no gender differences were observed, data were pooled across gender. Gaboxadol (THIP Hydrochloride, Sigma) and muscimol (Sigma) were diluted in normal saline, and flunitrazepam (Sigma) was diluted in a 22.5% (wt/vol) solution of 2-hydroxypropyl-β-cyclodextrin (Sigma). All drugs were administered into the intraperitoneal cavity in a volume of 0.01 mL per gram of body weight.

4.2.2 Fixed Speed Rotarod

The Ugo Basile 7650 (Varese, Italy) apparatus with a rod diameter of 6 cm that was rotating at a fixed speed of 6 rpm was used for all experiments measuring drug-induced ataxia. For description of mice used in ataxia studies, please see **Table 3**. Mice were acclimated to the apparatus by pre-training them on the rotarod 1-3 times on the day before each drug-induced ataxia experiment. Mice capable of walking on the rotarod for 180 seconds were used for drug-ataxia experiments. Mice were evaluated once again prior to drug injection. Mice were injected with drug and then placed on the rotarod every 15 minutes (gaboxadol and flunitrazepam) or every 30 minutes (muscimol) post-injection. The time a mouse was able to stay on the rotarod was recorded. Data were analyzed by repeated measures ANOVA.

Table 3. Description of mice used for drug-induced ataxia experiments

Knockout Strain	Drug	Dose (mg/kg)	Age (weeks)	Previous Treatment
α4	Gaboxadol	10	8-12	Drug Naïve
α4	Gaboxadol	15	9-13	10 mg/kg gaboxadol
α4	Flunitrazepam	2	10-14	10, 15 mg/kg gaboxadol
δ	Gaboxadol	10	8-9	Drug naïve
δ	Gaboxadol	15	9-11	10 mg/kg gaboxadol
δ	Gaboxadol	30	8-9	Drug naïve
α4	Muscimol	1.5	16-18	1.0 g/kg Ethanol (Plus Maze)
α4	Muscimol	2	8-9	Drug Naïve
α4	Muscimol	3	11-12	2.0 mg/kg muscimol
δ	Muscimol	1.5	8-16	Drug Naïve
δ	Muscimol	2	8-11	Drug Naïve
δ	Muscimol	3	10-16	2.0 mg/kg muscimol
α1	Muscimol	2	8-9	Drug Naïve
α1	Muscimol	3	9-11	2.0 mg/kg muscimol

4.2.3 Radiant Tail Flick Assay

A radiant tail-flick assay was used as described [245]. Briefly, drug naïve mice (age 8-14 weeks) were lightly restrained by placing them in a soft cloth pouch with the tail extended from one end. Focused light from a tail-flick analgesia meter (IITC Life Sciences, Woodland Hills, CA) was applied directly to a spot ~1 cm from the tip of the tail. Tail-flick latency was measured using a digital timer contained within the experimental apparatus. Baseline measurements were made using a moderate light intensity that yielded ~ 8 s basal response in prior experiments. The possibility of tissue damage was avoided by automatic shutoff of the light after 30 s if the mouse did not respond. On the first day, mice were weighed and tested for basal nociception. One day later, mice were injected with 10 mg/kg gaboxadol and tested for

latency to tail flick 30 min after injection. At least two measurements were taken and the mean value calculated for each mouse. Data were analyzed using a paired Student's t-test.

4.2.4 Open Field Assay

The open field assay was used to measure baseline activity and gaboxadol-induced and sedation. Drug naïve mice (8-10 weeks of age) were weighed and transported to the mouse behavioral room at least three hours prior to testing. All testing occurred between 12:00 and 17:00. Mice were injected with normal saline or 10 mg/kg gaboxadol. Twenty minutes after injection, mice were placed into a walled arena (43.2 cm x 43.2 cm x 30.5 cm) that was located in a sound attenuating cubicle (Med Associates, St. Albans, VT) and allowed to freely explore the activity chamber for 6 minutes. Distance traveled (cm) was measured automatically using an activity monitor. Data were analyzed using two way ANOVA with genotype and dose as the between-subject factors and Fisher's post-hoc test where appropriate.

4.3 RESULTS

4.3.1 Ataxic effects of gaboxadol in α4 KO mice

Recovery from ataxia induced by 10 or 15 mg/kg gaboxadol was measured using a fixed speed rotarod. KO mice were virtually insensitive to 10 mg/kg gaboxadol when compared to WT mice (**Fig. 9A**; repeated measures ANOVA by genotype; $F_{(1,31)} = 55$, $p<0.0001$). Ataxic response to 15

mg/kg gaboxadol was also greatly reduced in KO compared to WT mice (**Fig. 9B**; $F_{(1,28)} = 14$, $p<0.001$). Responses to flunitrazepam was tested as a negative control. This benzodiazepine is thought to exert its effects via synaptic $GABA_A$-Rs. We observed that KO mice did not differ significantly from WT littermates in recovery from ataxia following injection with 2 mg/kg flunitrazepam (**Fig. 9C**). These data have been published [237].

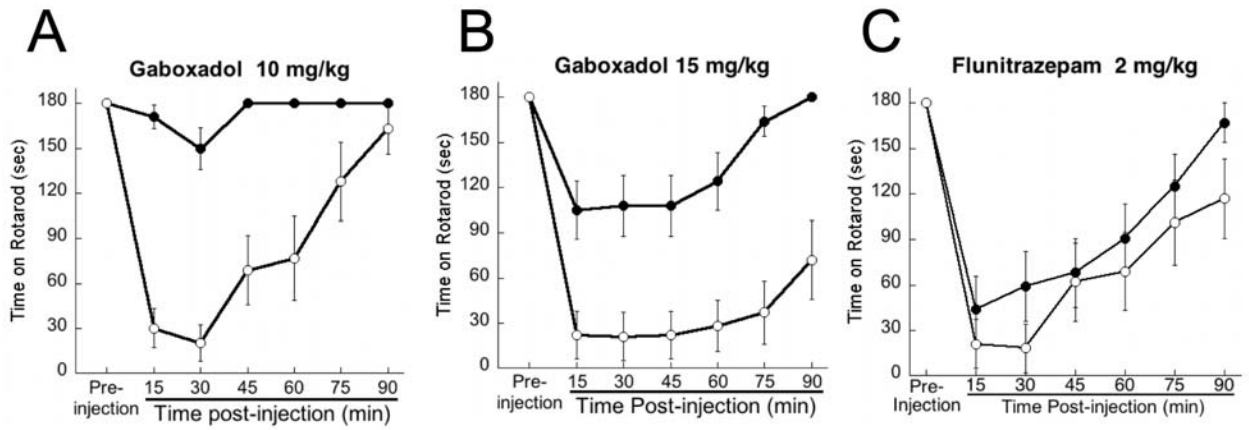


Figure 9. α4 KO mice have greatly reduced sensitivity to the ataxic effects of gaboxadol. The fixed speed rotarod measured gaboxadol's ataxic effects at (*A)* 10 mg/kg (n=19 KO, 14 WT) , (*B)* 15 mg/kg (n=18 KO, 12 WT). Ataxic effects of gaboxadol are dramatically reduced in alpha4 KO mice (black circles) compared to WT mice (white circles) at both 10 ($p < 0.0001$) and 15 mg/kg ($p < 0.001$). There were no differences between genotypes in ataxia induced by (C) flunitrazepam (n = 13 KO, 11 WT).

4.3.2 Ataxic effects of gaboxadol in δ KO mice

Recovery from ataxia induced by three different doses of gaboxadol (10, 15 or 30 mg/kg) was measured using a fixed speed rotarod (**Figs. 10A-C**). δ KO mice were completely insensitive to the ataxic effect of gaboxadol at 10 mg/kg [**Fig. 10A**; repeated measures ANOVA by genotype; ($F_{(1,45)} = 13.3$; $p < 0.01$)] and 15 mg/kg (**Fig. 10B**; $F_{(1,45)} = 6.7$; $p < 0.05$]. 30 mg/kg

impaired performance in δ KO mice on the rotarod, however, impairment was far less than wild-type mice [**Fig. 10C**; ($F_{(1,152)}$ = 15.1; $p < 0.005$]. These data demonstrate that δ KO mice have drastically reduced sensitivity to the ataxic effects of gaboxadol.

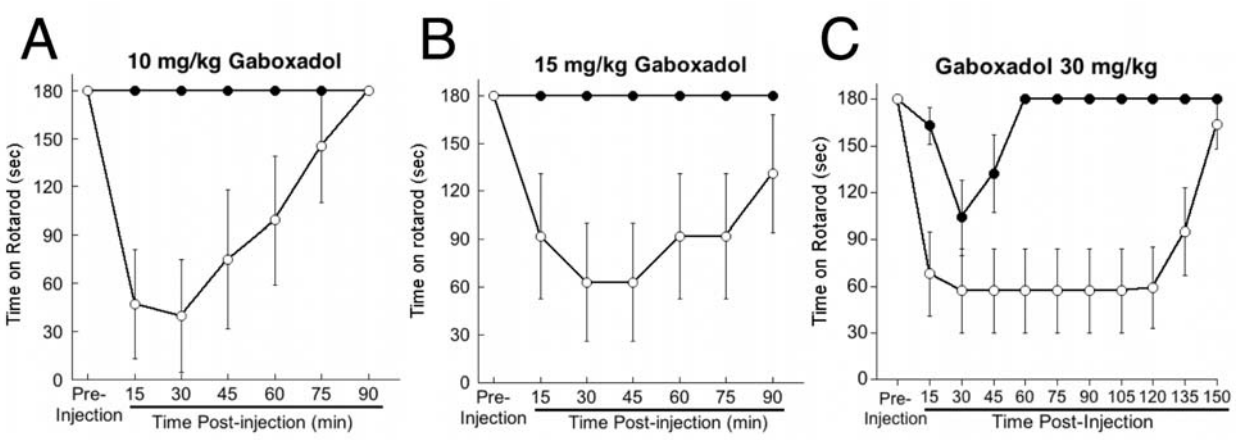


Figure 10. δ KO mice are largely insensitive to the ataxic effects of gaboxadol. Fixed speed rotarod measured the ataxic effects of gaboxadol at (A) 10 mg/kg (n = 6 KO, 5 WT), (B) 15 mg/kg (n = 6 KO and 5 WT) and (C) 30 mg/kg (n = 11 KO and 10 WT). Ataxic effects were either eliminated or greatly reduced in δ KO mice (black circles) compared to WT controls (white circles) at 10 mg/kg ($p < 0.01$), 15 mg/kg ($p < 0.05$) and at 30 mg/kg ($p < 0.005$).

4.3.3 Analgesic effects of gaboxadol in α4 KO mice

The radiant tail flick assay was used to measure thermal pain sensitivity and to study the analgesic effect of 10 mg/kg gaboxadol. WT and α4 KO mice did not differ in their basal thermal pain sensitivity, as measured by their latency to tail flick in the absence of drug (Fig. 11). In α4 KO mice, gaboxadol produced only a slight but significant ($p=0.05$) increase in tail flick latency compared to baseline. In contrast, gaboxadol markedly prolonged the latency to tail flick compared to basal responses in WT mice ($p<0.005$). These data indicate that α4 KO mice are

largely insensitive to the analgesic effect of gaboxadol as determined by the radiant tail flick assay. These data have been published [237].

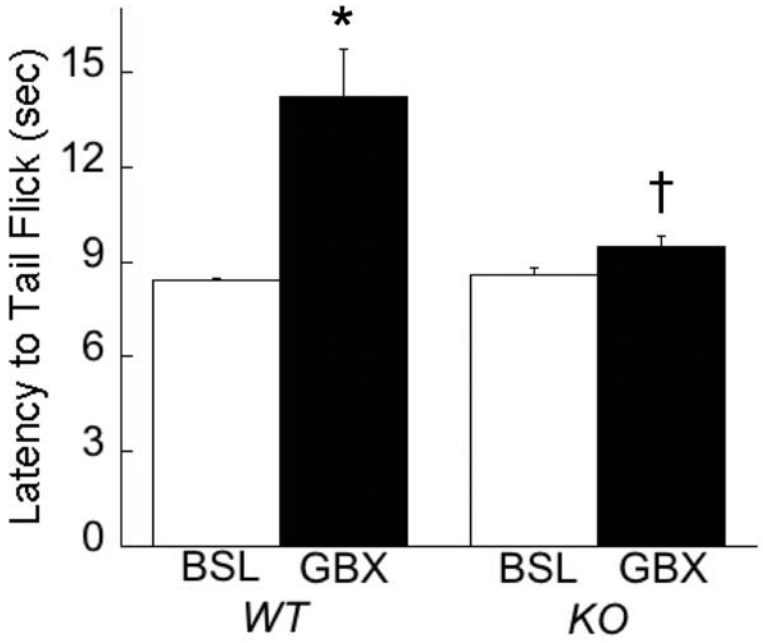


Figure 11. α4 KO mice are insensitive to the analgesic effect of gaboxadol. The radiant tail flick assay was used to measure the analgesic properties of 10 mg/kg gaboxadol (n=15 KO, 17 WT). Baseline latency to flick tail (BSL) or latency following gaboxadol injection (GBX) is displayed. Gaboxadol produced a marked analgesic effect in WT mice by increasing latency to flick tail (*, p<0.005) but only had a small but marginally significant (†, p=0.05) effect in KO mice.

4.3.4 Sedative effects of gaboxadol in α4 KO mice

To measure the sedative effects of gaboxadol, total locomotor activity was recorded in an open field assay in WT and α4 KO mice 20 min after treatment with either saline or 10 mg/kg gaboxadol (**Fig. 12**). Two way ANOVA revealed an effect of treatment ($F_{1,28} = 6$, $p<0.05$), a

genotype by treatment interaction ($F_{1,28}$; $p<0.05$), but no effect of genotype. Post hoc analysis revealed no difference in total activity between WT and α4 KO mice treated with saline. Treatment of WT mice with gaboxadol markedly decreased locomotor activity ($p<0.02$). In contrast, treatment of α4 KO mice with gaboxadol had no significant effect on activity. Thus, deletion of the α4 subunit of the $GABA_A$-R eliminated the sedative effects of gaboxadol as determined in the open field assay. These data have been published [237].

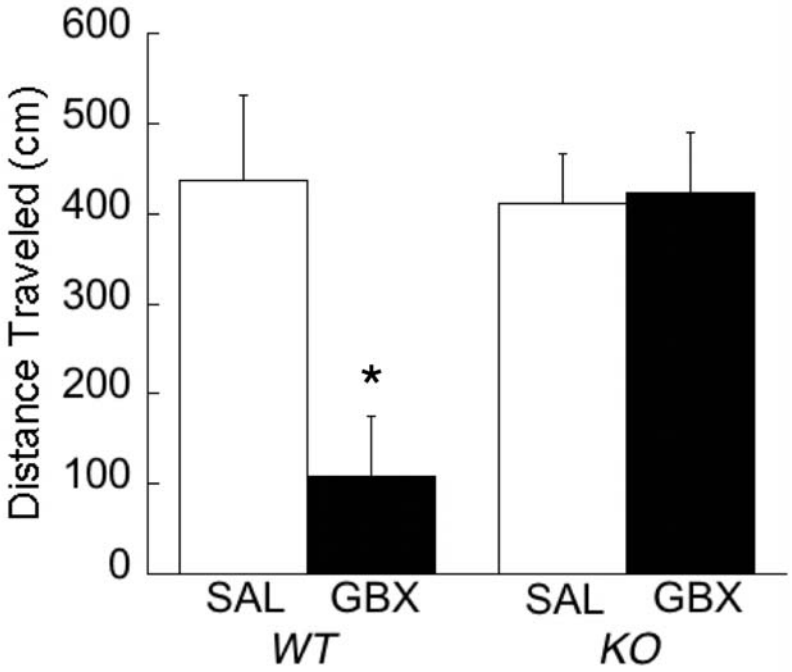


Figure 12. α4 KO mice are insensitive to the sedative effect of gaboxadol. The open field assay was used to measure the sedative effect of 10 mg/kg gaboxadol (n=7-10 mice / genotype / treatment). Total locomotor activity after injection with saline (SAL) or gaboxadol (GBX) is displayed. Gaboxadol depressed locomotor activity in WT mice (*, p<0.02), but had no effect in KO mice. No genotypic differences were observed between saline treated groups.

4.3.5 Ataxic effect of muscimol in α4 KO mice

Recovery from ataxia induced by 1.5, 2.0 and 3.0 mg/kg muscimol was measured by fixed speed rotarod (**Fig. 13**). α4 KO mice were significantly less sensitive to 1.5 mg/kg [**Fig. 13A**; repeated measures ANOVA ($F_{(1,64)} = 4.6$, $p < 0.05$)], 2.0 mg/kg [**Fig. 13B**; $F_{(1,100)} = 8.3$; $p < 0.05$], and 3.0 mg/kg [**Fig. 13C**; $F_{(1,100)} = 6.1$; $p < 0.05$]. There were no significant effects of gender ($p > 0.05$) at any of the three doses and therefore data from males and females were collapsed in the analysis. However, at 2.0 mg/kg, there was a trend toward an effect of gender [$F_{(1,100)} = 2.8$, $0.05 < p < 0.10$] with females possibly more sensitive to muscimol than males. These data demonstrate the reduced sensitivity of α4 KO mice to the ataxic effect of muscimol.

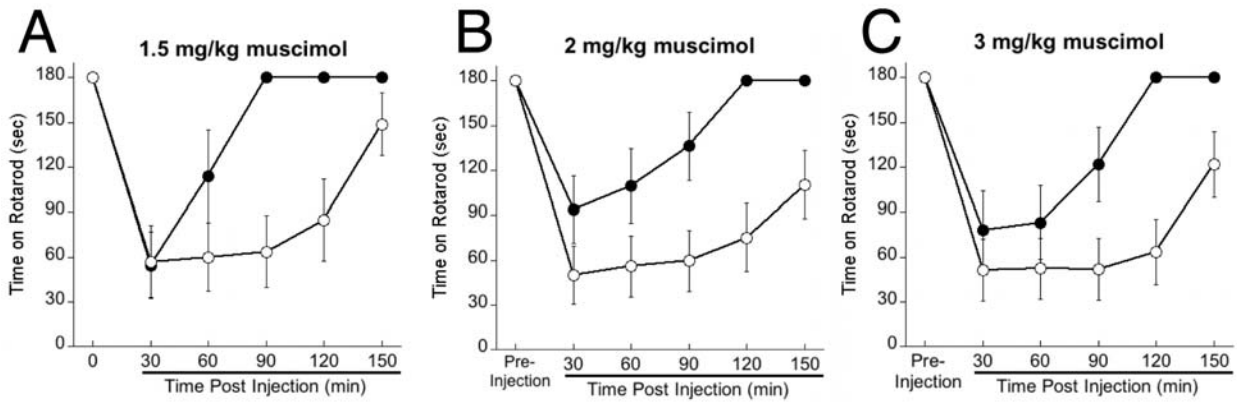


Figure 13. α4 KO mice are less sensitive to the ataxic effects of muscimol. The fixed speed rotarod measured muscimol's ataxic effects at 1.5 mg/kg (n = 7 KO and 11 WT) (A), 2.0 mg/kg (n = 12 KO and 15 WT) (B), and 3.0 mg/kg (n = 12 KO and 15 WT) (C). Ataxic effects of muscimol are reduced in alpha4 KO mice (black circles) compared with WT mice (white circles) at 1.5 ($p < 0.05$), 2.0 ($p < 0.05$), and 3.0 mg/kg ($p < 0.05$).

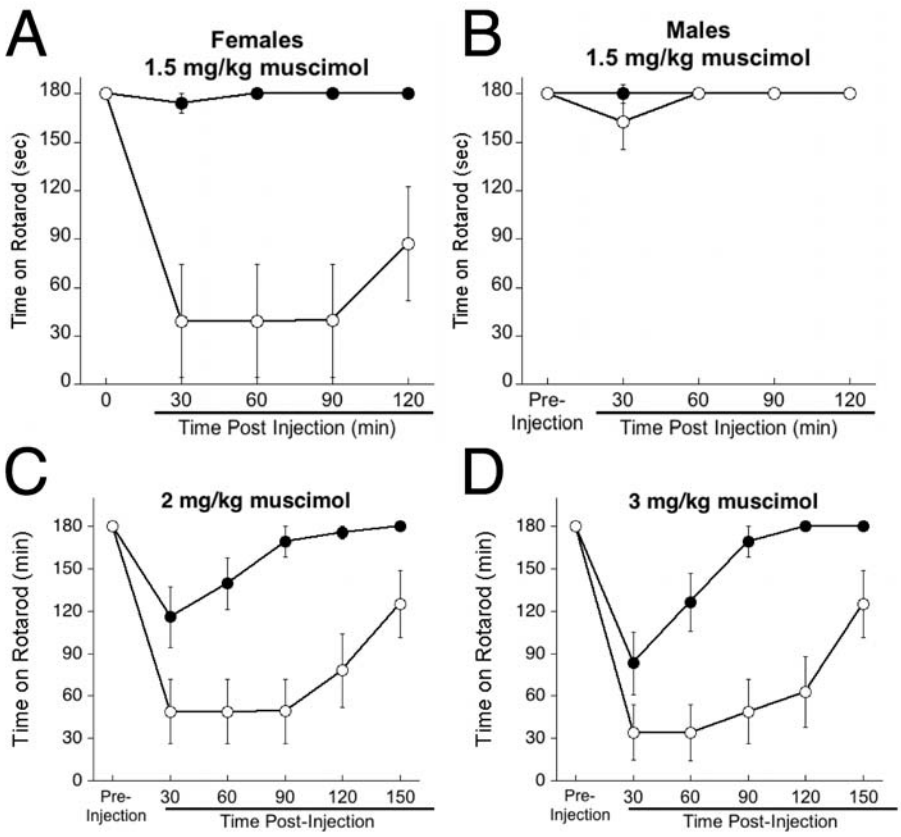


Figure 14. δ KO mice are less sensitive to the ataxic effects of muscimol. The fixed speed rotarod measured muscimol's ataxic effects in δ KO mice (black circles) and WT mice (white circles). At 1.5 mg/kg, there was a significant effect of gender ($p < 0.05$) and therefore, data were split between (A) females and (B) males. Ataxic effects of 1.5 mg/kg in female delta KO mice were greatly reduced ($p < 0.01$) compared to female WT mice (n = 5 KO and 5 WT). Male mice were not affected by muscimol at this dose (n = 3 KO and 4 WT). δ KO mice were less sensitive to the ataxic effects of muscimol at (C) 2.0 mg/kg ($p < 0.001$; n = 16 KO and 12 WT) and (D) 3.0 mg/kg muscimol ($p < 0.0001$; n = 16 KO and 12 WT).

4.3.6 Ataxic effects of muscimol in δ KO mice

Recovery from ataxia induced by 1.5, 2.0 and 3.0 mg/kg muscimol was measured by fixed speed rotarod (Fig. 14). There was a significant effect of gender at 1.5 mg/kg ($F_{(1,45)} = 4.9$, $p < 0.05$). Analysis of data split between genders, revealed a significant effect of genotype in

female mice (**Fig. 14A**; $p < 0.01$) but not male mice (**Fig. 14B**). Neither δ WT or KO males were affected by 1.5 mg/kg muscimol. At 2.0 and 3.0 mg/kg, no effect of gender was observed. δ KO mice recovered faster than WT following 2.0 mg/kg (**Fig. 14C**; $F_{(1,104)} = 17.8$, $p < 0.001$), and 3.0 mg/kg (**Fig. 14D**; $F_{(1,104)} = 21.5$, $p < 0.0001$). These data demonstrate that δ KO mice were less sensitive to the ataxic effects of muscimol.

4.3.7 Ataxic effects of muscimol in α1 KO mice

Recovery from muscimol induced ataxia was assessed in α1 KO mice and their WT littermate controls. Recovery from ataxia induced by 2.0 (Fig. 15A) and 3.0 mg/kg (Fig. 15B) muscimol was not different between α1 KO and WT mice.

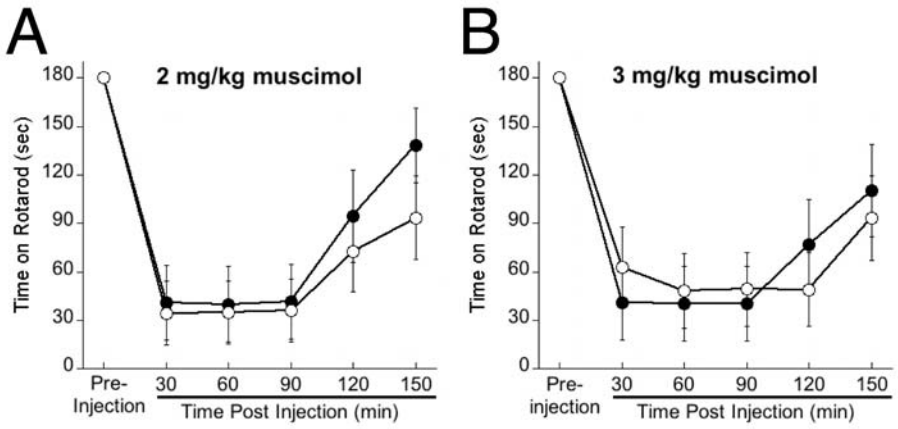


Figure 15. α1 knockout mice are equally sensitive as WT mice to the ataxic effects of muscimol. No differences in muscimol induced ataxia between α1 knockout mice (black circles) and WT littermate controls (white circles) at 2.0 mg/kg (A; n = 10 KO and 12 WT) and 3.0 mg/kg (B; n = 10 KO and 12 WT).

4.4 DISCUSSION

4.4.1 Extrasynaptic $GABA_A$-Rs in the behavioral effects of gaboxadol

Previous studies in $GABA_A$-R α4 KO mice ([237], Appendix C and D) demonstrated: 1) a reduction/elimination of tonic inhibition in the dentate gyrus and thalamus, and 2) insensitivity to gaboxadol at doses that enhance tonic currents in thalamic relay neurons of the VB. Here, we studied the role of extrasynaptic $GABA_A$-Rs in the behavioral effects of gaboxadol. We tested α4 KO and δ KO mice in their ataxic, analgesic, and sedative responses to gaboxadol. These studies found that both α4 KO and δ KO mice were largely insensitive to the behavioral effects of gaboxadol.

The ataxic effect of gaboxadol was measured using the rotarod assay. Under our experimental parameters, the performance of α4 WT mice on the rotarod was highly impaired by both 10 mg/kg and 15 mg/kg gaboxadol. In contrast, α4 KO mice were nearly insensitive to this effect, although a modest inhibitory effect remained at 15 mg/kg. The ataxic effects of gaboxadol in δ KO mice were also muted. δ KO mice were completely insensitive to both 10 mg/kg and 15 mg/kg gaboxadol. At 30 mg/kg, KO mice were affected but recovered far more quickly than δ WT mice.

Clearly, both α4 KO and δ KO mice were largely insensitive to the ataxic effects of gaboxadol. Though it is difficult to compare results across strains, our results indicate that δ KO mice are even more resistant to gaboxadol than α4 KO mice. δ KO mice were completely insensitive to gaboxadol's ataxic effects at both 10 and 15 mg/kg, while respectively, α4 KO mice were either barely or moderately effected by these same doses. The seemingly greater insensitivity in δ KO is not surprising given that gaboxadol is known to act as a potent agonist of

$GABA_A$-Rs that contain α4/δ or α6/δ [70]. Therefore α4/δ $GABA_A$-Rs are only a subset of δ containing $GABA_A$-Rs. In one sense, the observation that α4 KO mice were largely insensitive to gaboxadol-induced ataxia might seem surprising because performance on the rotarod is very sensitive to drugs that influence cerebellar activity. Of the $GABA_A$-Rs containing the δ subunit, the α6 subunit is highly expressed in the cerebellum but α4 is not [66]. One explanation could involve neurons that project from the cerebellum to thalamus; it is possible that potentiation of tonic current by gaboxadol reduces the activity of cerebello-thalamic-cortical networks crucial for balance and coordination. It is also possible that ataxia produced by gaboxadol is mediated outside of the cerebellum; α4/δ $GABA_A$-R populations in the striatum, thalamus and motor cortex would be the most obvious possibilities. Future studies using tissue-specific α4 KO may be informative in this regard.

Gaboxadol is also known to possess analgesic activity [138, 246], and this effect was also seen in our experiments using the radiant tail flick assay. Once again, the analgesic effect of a 10 mg/kg dose was significantly reduced/eliminated in the KO mice. These experiments indicate that the analgesic properties of gaboxadol are also mediated by $GABA_A$-Rs containing α4 subunits. The location of the α4 containing $GABA_A$-Rs responsible for this effect is unknown, although the receptor population in the thalamus is one possibility. It should be pointed out that the tail flick assay could be potentially confounded by motor effects of gaboxadol, so this phenomenon will have to be studied further in these mice using additional tests. It may be necessary to test α4 KO mice for analgesic properties either with non-sedative doses of gaboxadol or with other measures of analgesia such as the hot plate, Hargreaves test or von Frey assay.

α4 KO mice were tested for the sedative/ motor impairing effects of gaboxadol by

measuring locomotor activity in an open field. α4 WT mice showed a decrease in locomotor activity following i.p. treatment with 10 mg/kg gaboxadol. α4 KO mice, in contrast, were not impaired by gaboxadol; they had similar activity levels as WT and KO mice that were treated with saline. Recently, similar experiments in δ KO showed that δ KO mice are also insensitive to gaboxadol's effects in an open field [238]. Together, these results suggest a critical role for extrasynaptic α4/δ receptors in mediating the sedative/motor impairing effects of gaboxadol.

Thus far, studies of α4 knockout mice have found that: 1) tonic inhibitory currents are reduced/eliminated in dentate gyrus and thalamus; 2) thalamic VB neurons were completely insensitive to 100-300 nM gaboxadol, a dose that in WT mice produces robust potentiation of tonic currents but no effect on synaptic currents; and now, 3) that behavioral effects of gaboxadol are largely absent. These cellular and behavioral findings are mirrored in studies using δ KO mice [109, 238, 240, 247]. The combined results from these two mouse strains suggest that α4/δ $GABA_A$-Rs mediate a tonic current that is highly sensitive to potentiation by gaboxadol, and that potentiation of this tonic current by gaboxadol is responsible for the behavioral effects of this drug.

GBX is known to shorten the latency to sleep and to enhance the quality of sleep in man [159, 248, 249] and to produce sedation, analgesia, loss of motor co-ordination and loss of righting reflex in rodents [250, 251], but the mechanism by which this occurs is unknown. Relay neurons within the thalamus are known to show alterations in firing patterns during the transitions between the awake and the sleeping states [252, 253]. In collaboration with Neil Harrison's laboratory, we have shown the ability of gaboxadol to hyperpolarize these neurons in α4 WT neurons but not α4 KO. Therefore, there is a strong association between the sedative and motor effects of gaboxadol and the ability of the drug to hyperpolarize thalamic relay neurons.

One possibility is that gaboxadol is able to hyperpolarize these neurons sufficiently to induce the transition between silent or tonic firing modes and burst firing mode that is implicated in the onset of slow-wave sleep [254]. One way to test this is by behavioral analysis following local injection of gaboxadol into the thalamus.

4.4.2 Extrasynaptic $GABA_A$-Rs in behavioral responses to muscimol

The insensitivity of α4 and δ KO mice to gaboxadol provided the impetus to study the behavioral effects of muscimol, another direct $GABA_A$-R agonist structurally similar to gaboxadol. Ataxic responses to muscimol were measured using the rotarod. We observed that α4 or δ KO but not α1 KO mice were less sensitive to muscimol-induced ataxia.

α4 KO mice were significantly less sensitive to muscimol than WT mice at three doses ranging from 1.5 - 3.0 mg/kg. At the same doses, δ KO mice were also significantly less sensitive to muscimol than WT littermates. Reduced sensitivity to muscimol in α4 and δ KO mice is consistent with in vitro studies of $GABA_A$-Rs and molecular pharmacological studies using these knockout lines. Muscimol has a 40% greater maximal effect on extrasynaptic $GABA_A$-Rs than synaptic $GABA_A$-Rs [72, 144], although this difference in magnitude is much greater with gaboxadol (200%-300%) [72]. The fact that gaboxadol has greater specificity to extrasynaptic $GABA_A$-Rs than muscimol may explain why α4 KO and δ KO mice were nearly insensitive to gaboxadol but still displayed modest impairment by muscimol. Additionally, binding studies have found that δ KO mice have a greatly reduced number of high-affinity [^{3}H]-muscimol sites as measured by *in situ* autoradiography [147]. A similar reduction has also been observed in α4 KO mice (D. Chandra and E.R. Korpi, unpublished observations). Therefore, reduced behavioral sensitivity of α4 KO and δ KO mice may be due to loss of high affinity

binding sites.

Mice lacking the $GABA_A$-R α1 subunit were equally impaired by muscimol as their WT littermate controls. These results are interesting in light of previous molecular characterization of α1 KO mice showing that they have: 1) a 55% reduction in total $GABA_A$-Rs, 2) 35% reduced muscimol stimulated chloride uptake in cortical neurosynaptosomes, and 3) 55% reduced [^{3}H]-muscimol binding in cerebellar homogenates [187]. Unchanged behavioral sensitivity to muscimol in α1 KO mice is also interesting given that muscimol is a full agonist at α1β2γ2, whereas gaboxadol is only a partial agonist [70, 72, 144], and that α1 containing receptors are widely expressed throughout the brain, in far greater number than $GABA_A$-Rs contiaing α4 or δ [77, 78, 255]. It should be noted, however, that *in situ* autoradiography showed no differences in high affinity [^{3}H] muscimol binding in a conditional α1 knockout mouse model [229] unlike δ KO mice, which showed a large reduction [147]. Therefore, it may be possible that 1.5 – 3.0 mg/kg muscimol exerts its behavioral effects at these high affinity agonist binding sites. Another possibility could be that muscimol-induced ataxia is mediated by non-α1 synaptic $GABA_A$-Rs.

We have observed that α4 and δ KO mice have reduced sensitivity to muscimol suggesting that extrasynaptic $GABA_A$-Rs are important for the behavioral effects of this drug. The reduction in muscimol-induced ataxia is similar, but less dramatic, compared to the virtual elimination of gaboxadol-induced ataxia in these knockout lines. Similarly, it might be possible that α4 KO mice are less sensitive to the analgesic and sedative properties of muscimol like they were with respect to gaboxadol. It is also possible that the cellular mechanisms of these two drugs may be similar, e.g. via potentiation of tonic inhibitor current. Therefore, it would be worthwhile to study effects of muscimol in tonic and phasic inhibition in thalamic relay neurons of the VB. I hypothesize that tonic current in VB neurons would be enhanced by low

concentrations of muscimol that do not enhance synaptic currents. Finally, the similarities in responses to muscimol and gaboxadol indicate that the ataxic effects of other direct $GABA_A$-R agonists such as, thiomuscimol and isoguavicine [72, 144] may also be reduced in α4 and δ KO mice.

4.4.3 Summary

In summary, extrasynaptic $GABA_A$-Rs mediate tonic inhibition in the thalamus and dentate gyrus and mediate the ataxic, analgesic, and sedative effects of gaboxadol as well as the ataxic effects of muscimol. Potentiation of $GABA_A$-R tonic currents in various brain regions appear to be a mechanism of action that is shared with clinically used drugs including the anesthetics isoflurane [115] and etomidate [176, 179]. While neither gaboxadol nor muscimol has been approved clinically, these findings suggest that direct $GABA_A$-R agonists that preferentially act on high affinity extrasynaptic $GABA_A$-Rs may someday be exploited for use as sedative or analgesic agents.

5.0 RESPONSES TO ACUTE ETHANOL IN GABA$_A$-R α4 KO MICE

5.1 INTRODUCTION

Despite the use of EtOH throughout human history for its anxiolytic and intoxicating effects, the precise molecular targets through which EtOH exerts these effects are not well understood. Many different brain circuits and signaling systems have been implicated in the behavioral actions of EtOH in rodents [5]. One likely set of targets is the family of γ-aminobutyric acid type A receptors (GABA$_A$-R), which mediate inhibition throughout the mammalian brain.

GABA$_A$-Rs are pentameric complexes that function as ligand-gated chloride ion channels. There are a variety of subunit families that make up GABA$_A$-Rs; a total of nineteen distinct subunits have been cloned, α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3 [43]. This diversity in GABA$_A$-R subunit composition results in substantial anatomical, functional and pharmacological heterogeneity. For example, GABA$_A$-Rs containing α1, α 2, or α 3, with β2/3 and γ2 are typically found at sub-synaptic sites, where they mediate fast synaptic inhibition. In contrast, GABA$_A$-Rs containing α4 or α6, with β2/3 and δ are typically found at extrasynaptic locations, where they mediate a tonic form of inhibition by virtue of their ability to respond to low concentrations of GABA. Recent studies suggest that these extrasynaptic GABA$_A$-R populations may be targets for the behavioral effects of EtOH.

A large number of studies have shown that $GABA_A$-Rs are sensitive to EtOH [e.g.,156, 158], but in most studies the concentration required to potentiate those receptors was often greater than 60 mM, a concentration much higher than that achieved during social drinking. In contrast, *in vitro* studies of recombinant $GABA_A$-Rs of the extrasynaptic subtypes showed that these receptors were potentiated by concentrations of EtOH that approximate those achieved by social drinking (<30 mM) [162]. In another set of studies, recombinant $GABA_A$-Rs containing α4 and δ were potentiated by 1-3 [159] or 3-10 [73] mM EtOH, concentrations achieved by about half a glass to one glass of wine, although different results have been reported by other groups working with the same subunits [74]. Finally, the EtOH behavioral antagonist Ro15-4513 inhibited EtOH action at α4/δ receptors [164] and EtOH blocked binding of [^{3}H]Ro15-4513 to α4β3δ receptors [165], although other groups have been unable to observe similar effects [256, 257].

In vivo behavioral experiments also suggest that α6/δ and α4/δ extrasynaptic $GABA_A$-Rs mediate behavioral effects of EtOH. Rats with the Q100 allelic variation of the α6 subunit of the $GABA_A$-R were more sensitive to the motor-incoordinating effects of EtOH compared to rats with α6 (R100) [162]. The same study found that 1) potentiation of tonic current by EtOH was significantly greater in the α6 expressing cerebellar granule cells of α6(Q100) rats than α6(R100) rats and 2) EtOH more robustly potentiated in vitro receptors containing α6(Q100)β3δ than α6(R100)β3δ [162]. In another study, progesterone-withdrawn rats that exhibited increased α4δ expression were more sensitive to low concentrations of EtOH both at the cellular and behavioral levels [159]. Finally, mice lacking the δ subunit of the $GABA_A$-R showed reduced potentiation of tonic inhibition by EtOH [163, 178] and exhibited multiple defects in behavioral responses to EtOH [169].

$GABA_A$-Rs containing α4 and δ subunits mediate tonic inhibition in the dentate gyrus and in thalamic relay neurons [110, 111, 176, 177, 237]. The α4 subunit is also expressed in the cortex, striatum and other brain areas [53, 66, 175]. Thus, there is reason to suspect that these receptors might be involved in a variety of behaviors that are influenced by alcohol.

In the present study, we investigated the behavioral effects of EtOH in a recently created strain of α4 subunit knockout (KO) mice. These mice were largely insensitive to some behavioral effects of the $GABA_A$-R agonist, gaboxadol, also known as THIP [237], previously shown to be relatively selective for extrasynaptic α4/δ $GABA_A$R's [116, 180]. The α4 KO mice also showed a substantial deficit in tonic inhibition in dentate gyrus and thalamus [237], and the enhancement of tonic current by EtOH was greatly reduced in dentate gyrus granule cells in these mice [225, 258]. We therefore hypothesized that those behavioral responses to EtOH that are mediated by α4-containing $GABA_A$-Rs would be diminished in α4 KO mice.

5.2 METHODS AND MATERIALS

5.2.1 Mice

Breeding pairs of α4 heterozygous mice [237] were used to produce α4 KO and WT littermate controls. All mice were of a mixed C57BL/6J and Strain 129S1/X1 genetic background from the F2-F6 generations. Mice were genotyped at weaning using Southern Blot analysis of tail DNA as previously described [237]. Mice were group housed, given free access to standard rodent chow and water, and maintained on a 12 h alternating light/dark schedule with

lights on at 7:30 AM. For all experiments, both male and female mice were used, but no gender differences were observed and data was pooled across gender, except as noted in the results. Each mouse was used for only a single behavioral assay, except as noted below. EtOH was purchased from Pharmco (Brookfield, CT) and was administered into the peritoneal cavity (i.p.). The Institutional Animal Care and Use Committees at the University of Pittsburgh and the University of California, Los Angeles approved all protocols.

5.2.2 Elevated Plus-Maze

The elevated plus maze was used to test baseline anxiety and activity, as well as the anxiolytic and locomotor stimulatory effect of EtOH, using previously described methods [259]. Briefly, 8-10 week old mice were weighed and transported to the mouse behavioral room one day prior to testing. All testing occurred between 10:00 and 13:00 under ambient room light. Mice were injected with normal saline or 1.0 g/kg EtOH ten minutes prior to testing. Each mouse was placed on the central platform of the maze, facing an open arm and allowed to freely explore the maze for 5 min. Open-arm and closed-arm entries and the cumulative time spent on the open and closed arms were recorded. A mouse was considered to be on the central platform or on an arm when all four paws were within its perimeter. The percent open-arm entries, total number of entries and percent time in open-arms were determined. Data were analyzed using two-way ANOVA, with genotype and dose as the between-subject factors.

5.2.3 Open Field Assay

The open field assay was used to measure baseline activity and EtOH -induced locomotor stimulation and sedation. Mice (8-11 weeks of age) were weighed and transported to the mouse behavioral room one day prior to testing. All testing occurred between 12:00 and 17:00. Mice were injected with normal saline, 1.0 g/kg EtOH, or 2.0 g/kg EtOH ten minutes prior to testing. Each mouse was placed in the center of a walled arena (43.2 cm X 43.2 cm X 30.5 cm) that was located in a sound attenuating cubicle (Med Associates, St. Albans, VT) and allowed to freely explore the activity chamber for 10 min. Distance traveled and the number of rearings were automatically recorded using an activity monitor. The number of rearings was defined as the number of breaks of a photobeam 8 cm above the floor of the arena. Data were analyzed using two-way ANOVA with genotype and dose as the between-subject factors and Fisher's post-hoc test where appropriate.

5.2.4 Fixed Speed Rotarod

Mice were trained on a fixed speed rotarod (Ugo Basile, Model 7650 with rod diameter of 6 cm, Stoelting Co., Wood Dale, IL) and training was considered complete when mice were able to remain on the rotarod for 180 seconds. After EtOH administration, each mouse was placed back on the rotarod and time spent on the rotarod was measured for up to 180 s at intervals listed below for 60 min post-injection. Three independent experiments were conducted at three different dosages of EtOH, 1.5 g/kg, 2.0 g/kg, and 2.5 g/kg. In one experiment, 8-11 week old drug naïve mice were trained on the rotarod spinning at 14 rpm, and then injected with 1.5 g/kg EtOH. Performance on the rotarod was measured every 15 minutes.

In another experiment, 8-12 week old EtOH naïve mice were trained on the rotarod spinning at 6 rpm and then injected with 2.0 g/kg EtOH. Performance on the rotarod was measured every 10 minutes. In the final experiment, the same mice that were injected with 2.0 g/kg were re-trained two weeks later on the rotarod spinning at 6 rpm and injected with 2.5 g/kg EtOH. Performance was measured every 15 minutes. Data were analyzed within each experiment using repeated measures ANOVA.

5.2.5 Loss of Righting Reflex Assay

Mice were tested for the sedative/hypnotic response induced by EtOH using the loss of righting reflex (LORR) assay. Mice (10-15 weeks of age) were injected with 3.5 g/kg EtOH and then monitored for LORR. Once this occurred, mice were placed on their backs in v-shaped troughs. Mice were monitored until they were able to right themselves three times in 30 s. The duration of LORR was the time elapsed between when they were placed in a supine position and when they were able to right themselves three times. A heat lamp and monitoring of rectal temperatures were used to ensure normothermia. Data were analyzed using an unpaired Student's t test.

5.2.6 Radiant Tail Flick Assay

The analgesic properties of ethanol are well documented [260, 261]. A radiant tail-flick assay was used to measure this effect [245]. Mice that had been tested in the open field assay more than two weeks prior were used for this assay. Briefly, mice (age 10-14 weeks) were lightly restrained by placing them in a soft cloth pouch with the tail extended from one end.

Focused light from a tail-flick analgesia meter (IITC Life Sciences, Woodland Hills, CA) was applied directly to a spot ~1 cm from the tip of the tail. Tail-flick latency was measured using a digital timer contained within the experimental apparatus. Baseline measurements were made using a moderate light intensity that would yield ~10 s basal response based on prior experiments. The possibility of tissue damage was avoided by automatic shutoff of the light after 30 s if the mouse did not respond. On the first day, mice were weighed and tested for basal nociception. One day later, mice were injected with 2 g/kg EtOH and tested for latency to tail flick 20 minutes after injection. At least two measurements were taken and the mean value calculated for each mouse. Data were analyzed using repeated measures ANOVA.

5.2.7 Screen Test and Hypothermia Test

The screen test and EtOH-induced hypothermia were assayed together on the same mice. Mice (age 10-16 weeks) that were tested on the open field assay two weeks prior were used for these studies. The screen test was carried out using an apparatus that was constructed similar to that described previously [262]. The screen test apparatus was a 5 mm^2 grid mounted in a plastic frame and positioned 60 cm above a padded table. The grid was supported by two vertical arms in such a manner that allowed the grid to rotate around an axis perpendicular to the supporting arms. Mice were weighed, injected with saline, and then placed on the screen while the screen was horizontal. The screen was then rotated 90 degrees over 3 s. Mice were given two trials to pass the criterion latency of remaining on the screen for 240 s. Three days later, the rectal temperature of each mouse was measured. Five minutes after temperature measurement, each mouse was injected with either 1.5 or 2.0 g/kg EtOH. Twenty-five minutes after EtOH injection, each mouse was assayed on the screen test apparatus for latency to fall. Animals that did not fall

after 240 s were given a latency score of 240. Thirty minutes after injection, rectal temperature was again measured. For both the screen and hypothermia tests, data were analyzed using ANOVA and Fisher's post hoc test.

5.2.8 EtOH Metabolism and Clearance

Mice (13-16 weeks of age) were injected with EtOH (3.5 g/kg) and blood samples were collected from the retro-orbital sinus at 30, 60, 90, and 120 min following injection. Blood Ethanol concentration (BEC) was determined as described previously [263]. Briefly, blood was collected in heparinized capillary tubes, then mixed with 3% perchloric acid, and centrifuged for 10 min at 10,000g at 4 °C. The EtOH concentration in supernatants was measured using spectrophotometry via an alcohol dehydrogenase assay. Clearance was calculated as the average slope of a linear regression of BEC versus time. An unpaired Student's t-test was used to make comparisons between genotypes.

5.3 RESULTS

5.3.1 Elevated Plus Maze

The elevated plus maze test was administered to examine baseline anxiety-like behavior and the anxiolytic and locomotor stimulatory effects of EtOH. There were no differences between WT and KO mice in basal performance (i.e., following saline injection) on the elevated plus maze. Saline treated WT and KO mice did not differ in total arm entries, percentage of

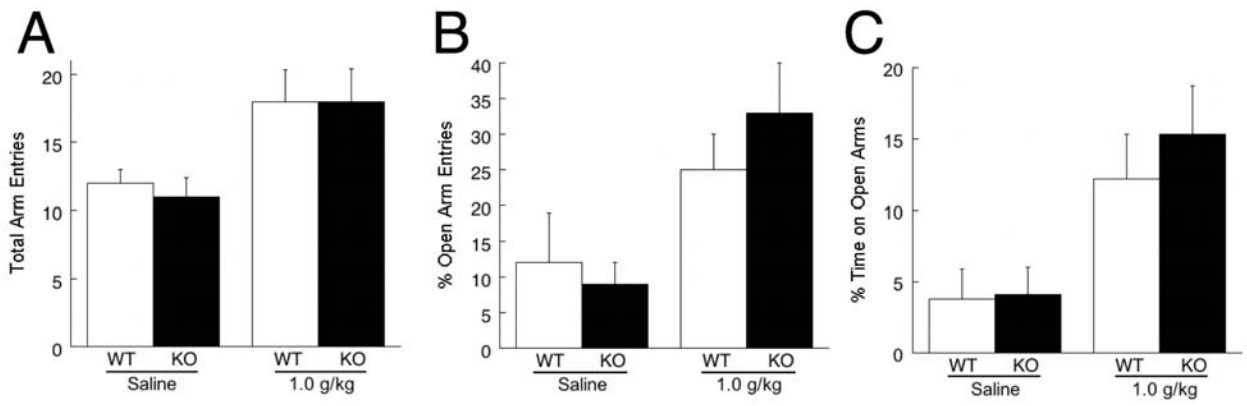


Figure 16. Evaluation of anxiety-like behavior and locomotor activity using the elevated plus maze. Total arm entries (A), percentage of open arm entries (B), and percentage of total time spent on open arms (C) are displayed. Saline or 1.0 g/kg EtOH was injected 10 min prior to testing. EtOH increased total arm entries ($p<0.005$), percentage of open arm entries ($p<0.005$), and percentage of time on open arms ($p<0.001$). However, there was no effect of genotype or interaction of genotype with treatment. Data represent mean ± SEM. n = 12-16 mice / genotype / treatment.

entries onto open arms, or percentage of time spent on open arms (**Fig. 16**). The locomotor stimulatory effect of EtOH was assessed by comparing the total number of arm entries in mice treated with saline versus those treated with EtOH (**Fig. 16A**). Although a 1.0 g/kg dose of

EtOH produced a significant increase in total entries [ANOVA: $F(1,52) = 10.3$, $p < 0.005$], there were no significant differences between KO and WT mice. The anxiolytic response to EtOH was assessed by comparing the percentage of open arm entries (Fig. 16B) and percentage of time spent on open arms (**Fig. 16C**) between mice treated with saline versus EtOH. A 1.0 g/kg dose of EtOH produced significant anxiolytic responses; EtOH treated mice showed an increase in open arm entries [$F(1,52) = 12.1$, $p < 0.005$] and time spent on open arms [$F(1,52) = 12.7$, $p < 0.001$]. However, KO mice did not differ from WT mice in their sensitivity to the anxiolytic effects of EtOH.

5.3.2 Open Field Assay

The open field assay was used to measure baseline activity as well as the locomotor stimulatory and sedative effects of EtOH. Analysis of total distance traveled revealed a significant main effect of EtOH treatment [$F_{(2,98)} = 17.4$, $p < 0.0001$] but there were no significant effects of genotype or the interaction of genotype and treatment (**Fig. 17A**). Distance traveled was increased by ~55% by 1.0 g/kg EtOH compared to mice treated with saline ($p < 0.0001$). Mice treated with 2.0 g/kg EtOH did not differ in distance traveled compared to saline controls.

Analysis of the number of rearings observed in the open field revealed a significant effect of gender [$F_{(1,102)} = 5.2$, $p < 0.05$], and so the data from male (**Fig. 17B**) and female mice (**Fig. 17C**) were analyzed separately. For both genders, there were significant main effects of EtOH treatment with respect to number of rearings observed [ANOVA: male, $F_{(2, 48)} = 19.6$, $p < 0.0001$; female, $F_{(2,44)} = 11$, $p < 0.0001$]. In males, 1.0 g/kg ($p < 0.05$) and 2.0 g/kg ($p < 0.0001$) doses reduced the number of rearings compared to saline-treated mice. In females, only 2.0 g/kg

EtOH reduced the number of rearings ($p < 0.0001$). There was no significant effect of genotype or interaction of genotype with dose on the number of rearings for either gender.

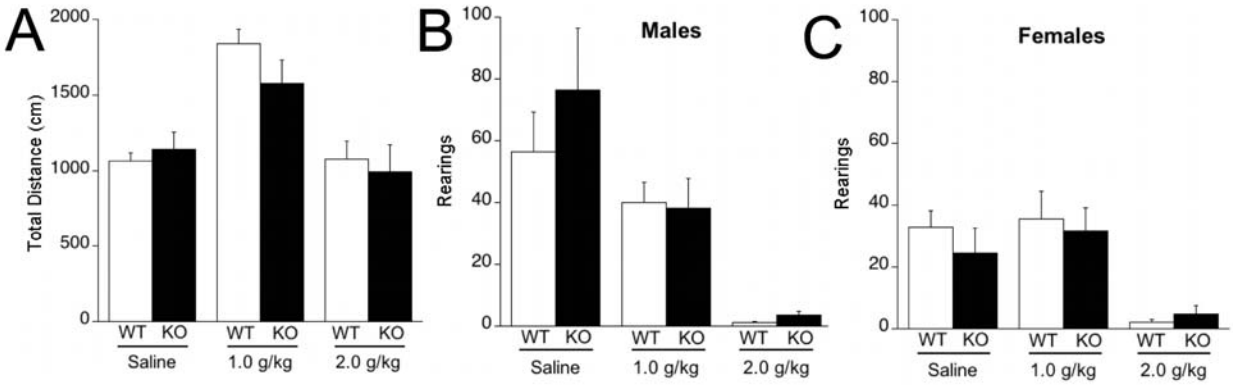


Figure 17. Evaluation of locomotor activity and rearing using the open field assay. Mice were injected with saline or EtOH 10 min prior to testing. (A) Total distance traveled by mice treated with 1.0 g/kg EtOH was greater than saline treated mice ($p<0.0001$), but there was no effect of genotype or interaction of genotype with treatment. Sample size was 16-18 mice / genotype / treatment. Analysis of the number of rearings revealed a significant effect of gender ($p < 0.05$) that warranted analysis of males and females separately. (B) The number of rearings in male mice was influenced by treatment ($p<0.0001$) but not by genotype. Both 1.0 g/kg ($p<0.05$) and 2.0 g/kg ($p<0.0001$) reduced the number of rearings compared to saline treatment. (C) The number of rearings in female mice was influenced by treatment ($p<0.0001$) but not by genotype. The 2.0 g/kg ($p<0.0001$) reduced the number of rearings compared to saline treatment. For panels B and C, n = 7-10 mice / genotype / treatment. Data represent mean ± SEM.

5.3.3 Fixed Speed Rotarod

Recovery from ataxia induced by three different doses of EtOH (1.5, 2.0 or 2.5 g/kg) was measured using a fixed speed rotarod in three separate experiments (Fig. 18). In all experiments, there was a significant effect of time [1.5 g/kg, $F(3, 48) = 21.2$, $p < 0.0001$; 2.0 g/kg, $F(4, 96) = 20.2$, $p < 0.0001$; 2.5 g/kg, $F(3,54) = 40.6$, $p < 0.0001$]. However, there were no significant effects of genotype or interaction of genotype with time for any of the doses tested.

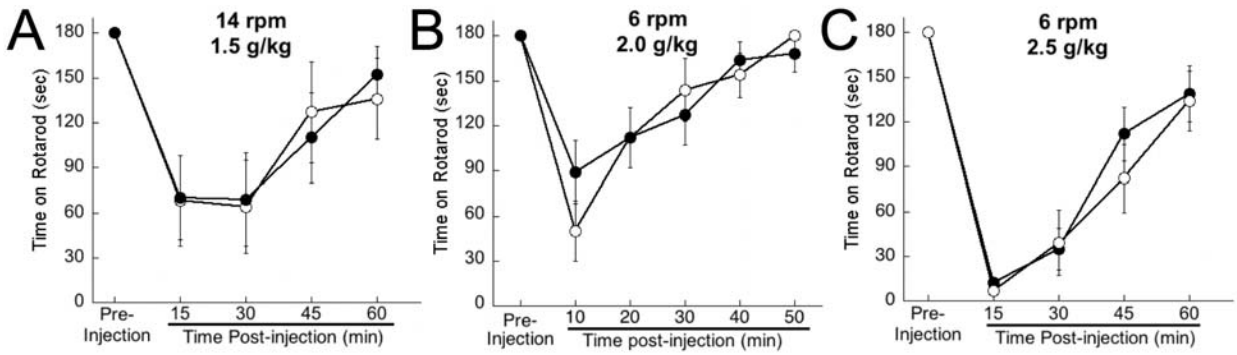


Figure 18. Fixed speed rotarod measured EtOH's ataxic effects at (A) 14 RPM, 1.5 g/kg (n = 9 KO and 9 WT), (B) 6 RPM, 2.0 g/kg (n = 15 KO and 11 WT), and (C) 6 RPM, 2.5 g/kg (n = 12 KO and 8 WT). Black circles represent KO mice and white circles represent WT mice. Data points are mean ± SEM. There was a significant effect of time at all doses tested ($p<0.0001$) but no genotypic effects were observed in EtOH-induced ataxia at any dose. Genotypes did not differ in the rate of learning the task during training sessions (data not shown).

5.3.4 Loss of Righting Reflex (LORR) Assay

The sedative/hypnotic effect of a 3.5g/kg dose of EtOH was determined using the LORR assay (**Fig. 19A**). WT and KO mice did not differ in the duration of LORR.

5.3.5 Radiant Tail Flick Assay

The radiant tail flick assay was used to measure thermal pain sensitivity and to study the analgesic effect of 2.0 g/kg EtOH (**Fig. 19B**). WT and KO mice did not differ in their basal thermal pain sensitivity, as measured by their latency to tail flick in the absence of drug. There was a significant main effect of EtOH treatment on the latency to tail flick [ANOVA: $F_{(1,21)}$ =

196, $p < 0.0001$]. However, there was no significant effect of genotype or interaction of genotype with EtOH. EtOH significantly prolonged the latency to tail flick compared with basal responses in both WT ($p < 0.0001$) and KO ($p < 0.0001$) mice.

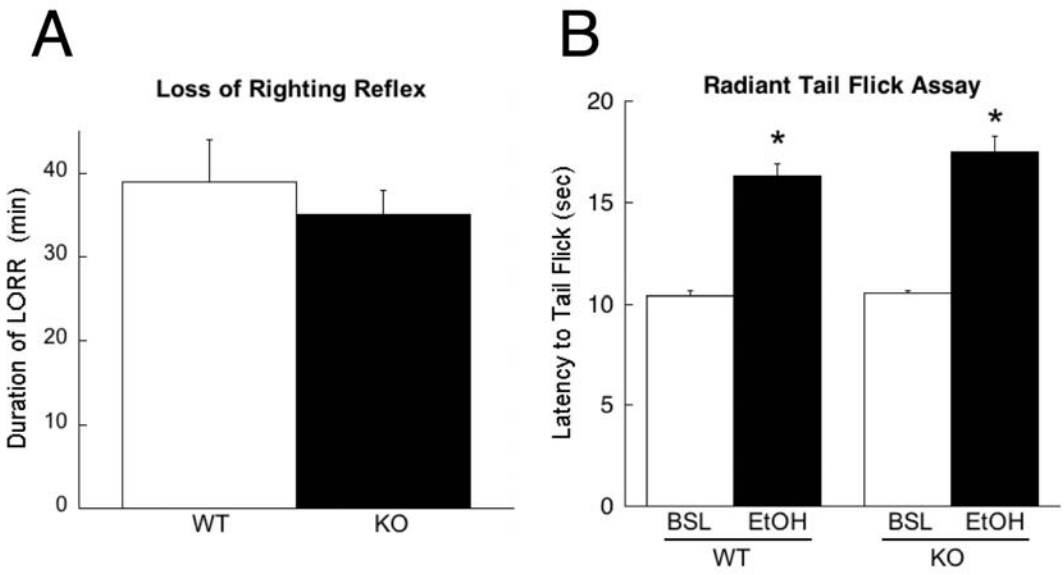


Figure 19. EtOH-induced sedative/hypnotic and analgesic effects. (A) EtOH-induced (3.5 g/kg) sedative/hypnotic effects measured by the duration of the LORR (n= 13 WT and 11 KO) was not different between genotypes. (B) EtOH-induced (2.0 g/kg) analgesia measured by a radiant tail flick assay (n = 13 WT and 10 KO). EtOH increased latency to tail flick over baseline (BSL) for both WT and KO mice. However, no significant effect of genotype was observed. All data represent mean ± SEM. *, $p < 0.0001$

5.3.6 Screen test

Results for the screen test are shown in **Fig. 20A**. Prior to being tested with EtOH, all mice were trained until they were able to remain on the screen for 240 s. There was a significant main effect of EtOH treatment on latency to fall [$F_{(1, 76)} = 10.6$, $p < 0.005$]. In contrast, there were no significant effects of genotype or interaction of genotype with EtOH on latency to fall from the screen. This demonstrated that both 1.5 and 2.0 g/kg EtOH impaired the ability to stay

on the vertical screen equally in both genotypes. This effect was dose-dependent as mice treated with 2.0 g/kg were more impaired than those treated with 1.5 g/kg ($p<0.005$).

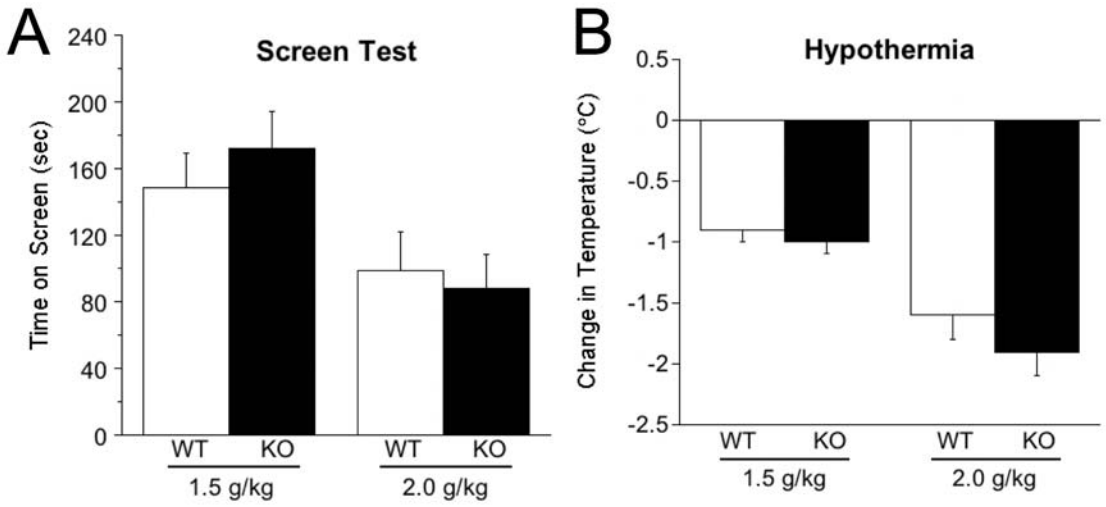


Figure 20. Screen test and EtOH-induced hypothermia. (A) All animals used achieved a baseline performance criterion of 240 sec. Injection of EtOH reduced latency to fall from screen ($p < 0.005$); this effect was dose dependent as 2.0 g/kg had a greater effect than 1.5 g/kg ($p < 0.005$). No effect of genotype was observed. (B) EtOH reduced body temperature ($p < 0.0001$), but no difference between genotype was observed. All data represent mean ± SEM. n = 20 / genotype / treatment.

5.3.7 Hypothermia test

EtOH -induced reductions in body temperature are displayed in **Figure 20B**. There were no differences in baseline body temperature between WT and KO mice (data not shown). There was a significant main effect of EtOH treatment on change in body temperature [$F_{(1,76)} = 34.0$, $p < 0.0001$] indicating that EtOH injection induced a hypothermic response. However, there was no significant main effect of genotype or interaction of genotype with dose in the hypothermic

effect of EtOH. The effect of EtOH was dose-dependent as 2.0 g/kg EtOH had a greater hypothermic effect than 1.5 g/kg ($p<0.0001$).

5.3.8 EtOH Metabolism and Clearance

To determine if KO mice differed from their WT littermates with respect to EtOH pharmacokinetics, BEC was measured every 30 min following 3.5 g/kg injection of EtOH. BECs did not differ significantly between genotypes at any timepoint measured. For example, at 90 min postinjection, BEC was 317 ± 27 mg/dl (n = 5) in WT animals compared to 333 ± 43 mg/dl (n = 5) in α4 KO animals. The rate of clearance of EtOH from the blood also did not differ between genotypes (WT, 2.5 ± 0.5 mg/dl/min, n = 5; KO, 2.4 ± 0.2, mg/dl/min, n = 5). Therefore, valid comparisons can be made between genotypes for acute behavioral responses to EtOH administration.

5.4 DISCUSSION

This study examined the effects of targeted inactivation of the gene encoding the α4 subunit of the $GABA_A$-R on the acute behavioral effects of moderate/high dose EtOH. α4 KO and WT littermate mice were tested on a wide-ranging battery of behavioral assays. Deletion of the α4 subunit of the $GABA_A$-R did not influence the behavioral responses to acute administration of EtOH in the elevated plus maze, open field, fixed speed rotarod, LORR, radiant tail flick, screen test, or hypothermia assays.

The lack of an EtOH-induced behavioral phenotype in the α4 KO mice is surprising given the following. Recombinant $GABA_A$-Rs containing α4 and δ subunits are reported to be sensitive to low concentrations (<30 mM) of EtOH associated with social intoxication [73, 159]; however see: [74]. *In vivo,* these receptors are located extrasynaptically where they mediate tonic inhibition that is also sensitive to moderate concentrations of EtOH [163, 178]. This tonic current is reduced in α4 KO mice and is not potentiated by EtOH [225, 258]. Mutation of the closely related α4/δ containing $GABA_A$-Rs leads to EtOH-induced behavioral changes [162]. The EtOH antagonist, Ro15-4513, binds to α4/δ containing receptors and reverses the effects of EtOH [164, 165]; however see: [256, 257]. Lastly, α4 KO mice are largely insensitive to gaboxadol, a GABAergic drug whose effects are mediated by α4/δ containing $GABA_A$-Rs [237]. Despite these previous studies that strongly implicate α4 in EtOH action, our current results argue against a key role for α4 subunit-containing $GABA_A$-Rs in mediating EtOH-induced behavioral effects.

There are a number of possible explanations for the lack of an EtOH-induced acute behavioral phenotype in the α4 KO mice that must be considered. As with all knockout studies, compensatory mechanisms may mask the normal endogenous effect of a gene that has been knocked out. As mentioned above, α4 KO mice were less sensitive to EtOH at the cellular level; two independent studies demonstrated that EtOH potentiation of tonic inhibition was reduced in dentate gyrus granule cells of α4 KO mice [225, 258]. However, Liang et al. also unexpectedly observed a compensatory increase in synaptic sensitivity to EtOH contrasting equal synaptic enhancement of gaboxadol between KO and WT mice. This increase in synaptic potentiation by EtOH in α4 KO mice may mask the true contribution of the α4 subunit in mediating EtOH-induced behaviors. Compensation is not unprecedented in $GABA_A$-R subunit KO mice as

numerous compensatory changes have been observed in other $GABA_A$-R subunit mutants. Compensatory mechanisms have included alterations in other $GABA_A$-R subunits [186-190], organization of GABAergic circuits [264], neuronal architecture [265], and genes and proteins outside the $GABA_A$-R system [122, 266]. The molecular compensation in α4 KO mice is only beginning to be understood. For example, brain-region selective compensatory increases in γ2 subunit protein in α4 KO mice have been discovered [225].

It is also conceivable that no genotypic differences in EtOH -induced behaviors between α4 WT and KO mice were observed because the EtOH doses used were too high. The α4/δ-containing receptors may be selective targets for only very low dose effects of EtOH. In this study, we gave doses of EtOH of 1.0 – 3.5 g/kg that likely produce peak blood EtOH levels between 15-100 mM [267]. In contrast, recombinant receptors containing α4 and δ subunits can be potentiated *in vitro* by EtOH concentrations as low as 1–3 mM [73, 159]. To achieve low millimolar EtOH concentrations in mice might require injection of no more than 0.25 g/kg EtOH. Unfortunately, no behavioral tests in mice have been developed where there is a measurable response to such a low dose of EtOH. Therefore, understanding the role of α4 containing $GABA_A$-Rs in EtOH action may be dependent on the development of new EtOH -induced behavioral paradigms in rodents that are sensitive to BEC in the low millimolar range.

A third possible explanation is that α4 mediates the effects of EtOH on behaviors that were not assessed by our study. The behavioral effects of EtOH reported here involved anxiety, locomotor activity, motor coordination, thermal pain sensitivity, and hypothermia. It is possible that α4 may be involved in other effects of EtOH such as impairment of cognition (e.g., executive decision making, or learning and memory), habit formation, control of EtOH drinking behavior, development of tolerance or dependence, or seizure protection. These behavioral

endpoints will be examined in future studies in the α4 KO mice as some of these EtOH effects were altered by knockout of the δ subunit [169]. Examination of the role of α4 in mediating alcohol tolerance and dependence will be particularly interesting as α4 expression is robustly increased following chronic exposure to EtOH [e.g., 60, 61, 182, 268]. Studies using α4 KO mice may therefore lead to an understanding of the cause and effect relationships between increased α4 expression and EtOH tolerance and dependence.

We conclude that in contrast to an obligatory role of α4-containing $GABA_A$-Rs in gaboxadol action [237], these receptors are not essential for the behavioral effects of acutely administered moderate/high dose EtOH that were tested in this study. However, further study is required to completely understand the true contribution of α4 containing $GABA_A$-Rs to the entire spectrum of EtOH -induced behaviors.

6.0 SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

$GABA_A$-Rs containing the α4 subunit have attracted a great deal of attention because of its: 1) specific localization in the thalamus, dentate gyrus, cortex and striatum, 2) possible role in mediating tonic inhibition, 3) plasticity of expression in a number of pathophysiological and experimental conditions and 4) possible role in mediating the behavioral effects of several drugs including gaboxadol and ethanol. The last of the four, the behavioral mechanism of action of gaboxadol and ethanol, was the main focus of this thesis.

In order to study the physiological role of α4 containing $GABA_A$-Rs, mice with targeted mutations in the α4 gene were created (Chapter 3). Cre/lox, gene targeting and embryonic stem cell technologies were used to create both a global α4 knockout mouse strain as well as a floxed α4 strain for later use in conditional knockout models. The global α4 KO mice created here were overtly normal and indistinguishable from their WT littermate mice. To date, this is the only knockout strain of α4 that is available to the scientific community. This knockout line already has, and will continue to be a valuable tool for investigators studying α4 containing $GABA_A$-Rs.

From studying global α4 KO mice, it was found that 1) tonic inhibitory currents were reduced/eliminated in dentate gyrus and thalamus; 2) thalamic relay neurons of the ventrobasal complex were completely insensitive to 100-300 nM gaboxadol, a dose that in WT mice produced robust potentiation of tonic currents but no effect on synaptic currents; and 3) that

behavioral effects of gaboxadol were largely absent (Chapter 4). These cellular and behavioral findings are mirrored in studies using δ KO mice [109, 238, 240, 247]. The combined results from these two mouse strains suggest that α4/δ $GABA_A$-Rs mediate a tonic current that is highly sensitive to potentiation by gaboxadol, and that potentiation of this tonic current by gaboxadol is responsible for the behavioral effects of this drug. Potentiation of $GABA_A$-R tonic currents in various brain regions appear to be a mechanism of action that is shared with other sedative-hypnotic drugs including the anesthetics isoflurane [115] and etomidate [176, 179].

Arguably, the most intriguing hypothesis about α4/δ $GABA_A$-Rs is that they mediate the behavioral effects of ethanol. Several studies have suggested that, like gaboxadol, ethanol may produce its behavioral effects through potentiation of tonic inhibition [162] (see section 1.6.4). This idea is derived, in large part, from *in vitro* studies showing that recombinant α4/δ $GABA_A$-Rs are potentiated by concentrations of EtOH that approximate those achieved by social drinking (<30 mM) [162] [73]. In contrast, $GABA_A$-Rs that are found synaptically are also sensitive to EtOH, but at concentrations greater than 60 mM [e.g.,156, 158], a concentration that is perhaps not physiologically relevant. It was therefore hypothesized that whichever behavioral responses to ethanol are mediated by α4-containing $GABA_A$-Rs would be diminished in α4 KO mice. As it turned out, α4 KO mice did not differ from WT littermates with respect to ethanol-induced changes in anxiety, locomotion, motor coordination, sedation, hypnosis, analgesia and thermoregulation (Chapter 5).

The lack of any difference in α4 KO mice in ethanol-induced behaviors starkly contrasts with their near insensitivity to gaboxadol. This dichotomy could lead to the conclusion that the ethanol-induced behaviors that were studied were not mediated by $GABA_A$-Rs containing α4 subunits. On one hand, this may not be surprising. After all, gaboxadol is a GABA-mimetic

drug that acts predominantly on extrasynaptic $GABA_A$-Rs with no reported action outside the GABA system. In contrast, ethanol is likely to have multiple protein targets outside of $GABA_A$-Rs [5]. Even though studies have found that extrasynaptic receptors are unique among $GABA_A$-Rs because they are potentiated by physiologically relevant concentrations of ethanol, it should be noted that other receptors outside the GABA system are also modulated by low doses of ethanol. Kainite receptors [269], corticotropin-releasing factor type 1 receptors [270], BK [271] and GIRK [261, 272] potassium ion channels are also sensitive to physiologically relevant ethanol concentrations (< 40 mM). Therefore, it might be possible that the selective loss of α4/δ mediated extrasynaptic inhibition did not disrupt the majority of important ethanol targets and therefore did not result in any changes in ethanol induced behaviors.

Despite the normal sensitivity to ethanol in α4 KO mice, it cannot be concluded that $GABA_A$-R α4 subtypes are not important for the behavioral effects of ethanol (see section 5.4). This is primarily due to molecular compensation that was observed in α4 KO mice. In physiological recordings from dentate granule cells, Liang et al. [225] found, as expected, that neurons from the α4 KO animals showed less overall tonic $GABA_A$-R mediated current and no significant potentiation of this current at concentrations of ethanol that reliably enhanced currents in WT mice. Unexpectedly, however, ethanol enhanced synaptic $GABA_A$-Rs function in α4 KO mice by ethanol concentrations that had no effect on neurons from WT mice. These increases in the synaptic sensitivity may be caused by a marked increase in expression of the γ2 subunit in both thalamus and hippocampus of α4 KO mice [225]. The synaptic supersensitivy to ethanol in α4 KO mice could have masked any changes in behavior that might have resulted from the lack of ethanol potentiation of tonic currents. Therefore, the only conclusion that can be drawn is that the $GABA_A$-R α4 subunit is <u>not essential</u> for the ethanol-induced behavioral endpoints that were

measured.

This compensatory change in α4 KO mice underscores a general limitation of global knockout mice in studying complex biological functions. Several other mouse strains lacking a particular $GABA_A$-R subunit have numerous compensatory changes in other $GABA_A$-R subunits [186-190], in organization of GABAergic circuits [264] and in proteins outside the $GABA_A$-R system [122]. Therefore, the compensatory effects resulting from a knockout of α4 needs to be better understood in order to make valid conclusions. Thus far, in addition to the upregulation in γ2 [225], there is an increase in α1 and α2 and a decrease in δ in the thalamus of α4 KO mice [226]. Other studies measuring changes in expression of other $GABA_A$-R subunits are underway. Additionally, gene array studies could be employed to quickly identify a multitude of genes that may be down or upregulated in α4 KO mice.

The possibility of compensatory effects following global knockout of α4 had been anticipated prior to commencing these studies. For this reason, Cre/lox technology was employed to create both a global knockout allele and also a "floxed" allele for conditional knockout studies. Theoretically, one way to reduce compensatory effects is to utilize an inducible knockout instead of a global knockout. Here, we studied ethanol-induced behaviors in α4 KO mice when they were at least eight weeks of age. Therefore, KO mice would have had plenty of time during its pre-natal, post-natal, youth, adolescence and early adulthood for their brains to compensate for the loss of extrasynaptic inhibition caused by the deletion of α4. In contrast, if floxed α4 mice were bred to an inducible Cre-recombinase expressing transgenic strain, knockout of α4 could be induced just a few days prior to behavioral testing. Induced α4 KO mice would have less time to compensate and perhaps the true contribution of α4 subunits in ethanol-induced behaviors could be assessed.

Another way to circumvent many the confounding compensatory changes in $GABA_A$-R α4 subunit knockout mice could be to create mice with a point mutation (knockin) in the α4 gene that makes them less (or more) sensitive to ethanol. The ideal knockin mouse would express α4 normally and α4 containing $GABA_A$-Rs would function normally in the absence of ethanol. Recently mice were created that had mutations in the transmemembrane region of the $GABA_A$-R α1 subunit that made them insensitive to ethanol with normal sensitivity to GABA [273]. Selective alterations in behavioral responses to ethanol were observed in this mouse strain [274]. Knockin of $GABA_A$-R subunits also have been successfully employed to study molecular mechanisms of anesthetics [275, 276] and benzodiazepines [134, 135, 277]. Studying α4 and ethanol using the knockin strategy is, of course, predicated on the identification of point mutations in α4 that produce either supersensitivity or insensitivty to ethanol. As yet, such a mutation in the α4 subunit has not been reported. However, a naturally occuring R100Q mutation in the closely related α6 subunit makes these $GABA_A$-Rs more sensitive to ethanol [162]. It could be possible that an analogous mutation in α4 may also result in receptors that are supersensitive to ethanol. Another possibility may be to mutate a transmembrane residue in α4 similar to that created in α1 that resulted in ethanol insensitive $GABA_A$-Rs [273]. Considerable effort (and some luck) is required to identify a specific residue in a $GABA_A$-R subunit that can substantially alter ethanol sensitivity without affecting overall function. However, the investement in time may be worthwhile; an α4 knockin mouse would likely have little (or no) compensation and therefore would be a more effective model in studying the role of α4 in the behavioral effects of ethanol.

Physiologic processes such as ethanol-induced behaviors may involve multiple gene products and therefore, knockout studies must take into account possible genetic background

variation. Several studies have found that different inbred mouse strains vary considerably in their responses to ethanol [262, 278]. The genetic background of α4 KO and their WT littermate controls was a random mixture of C57BL/6J and Strain 129 S1/X1. Therefore, with respect to ethanol sensitivity, there may be animal to animal variability that is independent of the α4 allele. This variability could have masked small differences in ethanol sensitivity between WT and KO. Ideally, α4 KO mice should have been backcrossed for at least 10 generations in order to determine the unique contribution of the α4 gene. Another confounding factor between WT and α4 KO mice is differences in the genes linked to the α4 locus. In KO (or f/f, See **Fig. 5A**) mice, the genes surrounding the α4 locus are Strain 129 derived whereas in WT (+/+) mice, they are C57BL/6J derived. Potential allelic differences in genes around the α4 locus may confound our observations. In this sense, control mice in α4 KO studies should have included both the homozygous floxed mice (F/F) in addition to WT mice (+/+) in order to control for the effects of linked genes. It is important to note, however, that while it would have been ideal to control for genetic background and linked gene effects in the studies presented in this dissertation, it would have required far more time and effort to do so.

At the outset of these experiments, a pharmacological approach for examining the significance of extrasynaptic $GABA_A$-Rs in ethanol action was not possible because specific inhibitors were not available. The studies presented here clearly demonstrate that the direct GABA agonists, gaboxadol and muscimol, preferentially activate extrasynaptic $GABA_A$-Rs partly because extrasynaptic $GABA_A$-Rs have a higher affinity for GABA. It is plausible that competitive $GABA_A$-R antagonists such as bicuculline or SR95531 may block extrasynaptic receptors at low doses but block all $GABA_A$-Rs at higher doses. In fact, SR95531 (20 μM) induced a depolarization in α4 WT but not in α4 KO thalamic relay neurons. Hypothetically, if

20 μM SR95531 were to have no effect on synaptic currents, then it could be used as a selective extrasynaptic $GABA_A$-R inhibitor. In this scenario, low concentrations of SR95531 could be coadministered with ethanol to understand the contribution of extrasynaptic receptors to ethanol induced behaviors.

In conclusion, the experiments presented here have unambiguously demonstrated the obligatory role of extrasynaptic $GABA_A$-Rs in the mechanism of action of gaboxadol. However, studies far beyond the scope of this dissertation are required to understand the true contributions of extrasynaptic $GABA_A$-Rs in ethanol induced behaviors. Defining the molecular targets that mediate the behavioral effects of ethanol has proven difficult and it might be unrealistic to think that one single target/receptor is highly important. However, if an "ethanol receptor" were ever identified, it would be an enormous discovery with broad clinical applications. Synthetic agonists of the "ethanol receptor" could mimic the positive effects of ethanol such as anxiolysis and cardioprotection. Such agonists could be used to treat alcohol withdrawal in a manner similar to which methadone treats symptoms of heroin withdrawal. Antagonists of an "ethanol receptor" could be used to reverse not only the motor and judgement impairing effects that lead to accidents, but also other systemic effects of alcohol poisoning that lead to coma and death. An antagonist could also be used to aid in the treatment of alcohol tolerance and dependence. For these reason, defining the molecular targets that mediate ethanol action is, and will continue to be, an extremely important undertaking.

APPENDIX A

SOUTHERN BLOT PROBE INFORMATION

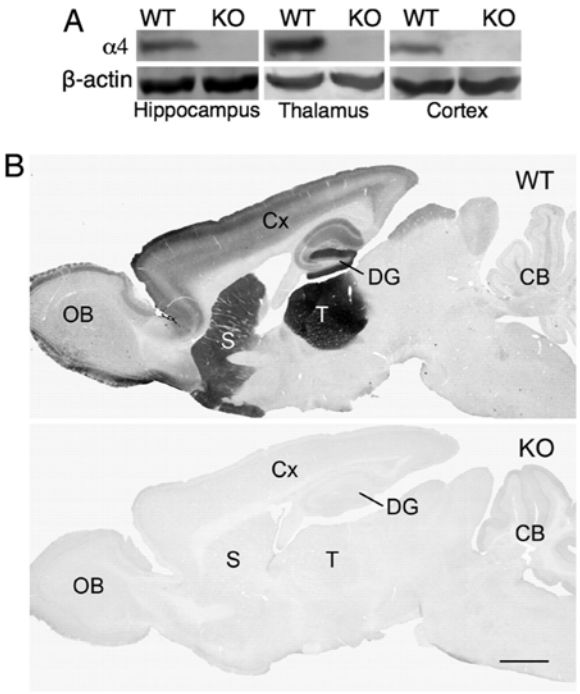
5' (see Fig. 2)- probe is external to targeting construct of a4 CKO and a4R100Q Knockin construct. This probe was used for stem cell screening. See a4B-254 for instructions on how to make. Briefly, digest pa4Intron2 plasmid with KpnI and EcoR1. Probe is 747 bp.

3' (Fig. 2) aka Exon 7 is external to targeting construct of a4 CKO and a4R100Q knockin construct-used for stem cell screening. See a4B-88 for instructions on how to make. Briefly, need to double digest pa4-78 with NheI and ClaI. Probe is ~500 bp

E (Fig. 5) probe is known as **a4KO probe**. This probe genotypes a4KO mice. This probe was created by amplifying a4cDNA plasmid (a4-17) with two primers detailed on page a4B-231. Probe is ~220 bp

APPENDIX B

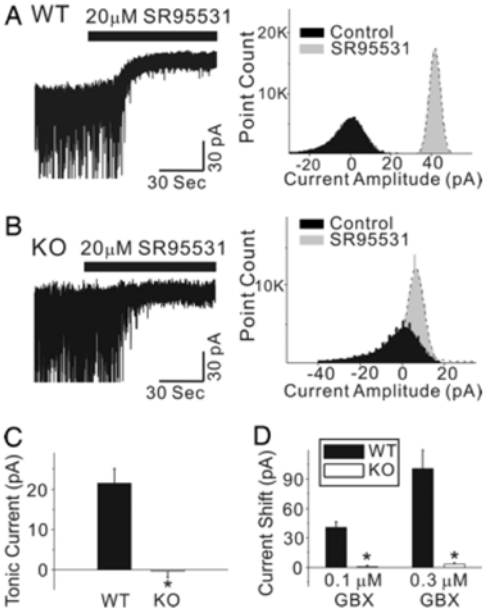
GABA(A) -R α4 PROTEIN IS ABSENT IN KO MICE



$GABA_A$-R α4 protein is absent in KO mice. (*A*) Western blot analysis of hippocampal, thalamic, and cortical membranes from WT and KO mice. The 67-kDa immunoreactive 4 protein present in WT samples is completely absent from KO samples. Stripped blots probed for -actin show equal loading of samples. (*B*) α4 Subunit immunoreactivity in sagittal sections from WT and KO mouse brain. In WT mice, α4 labeling is highest in the thalamus (T), moderate in the molecular layer of the dentate gyrus (DG) and striatum (S), and slightly lower in the outer layers of the cerebral cortex (Cx) and the external plexiform layer of the olfactory bulb (OB). α4 Labeling is essentially absent from the cerebellum (CB). No specific labeling is evident in KO mouse brain. Published in Chandra et al. Proc. Natl. Acad. Sci. 2006, 103: 15230-35.

APPENDIX C

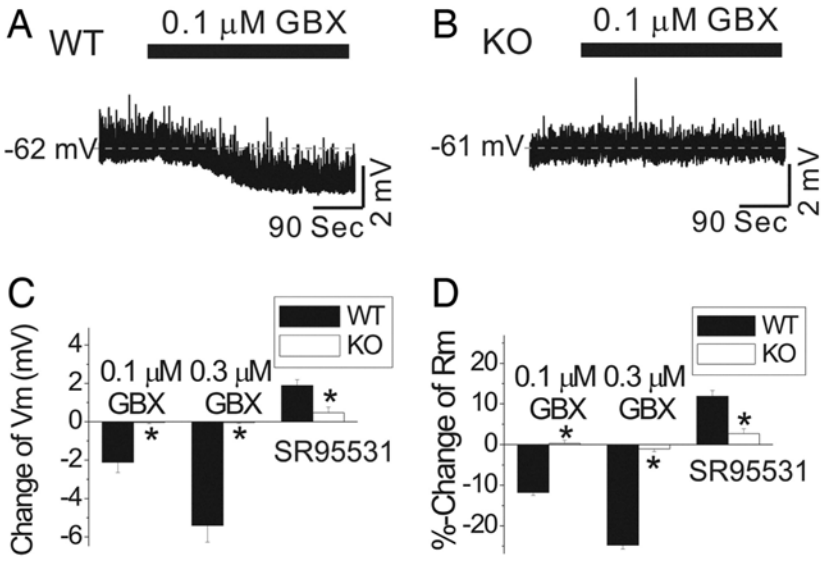
LOSS OF TONIC INHIBITION IN GABRA4 KO MICE



Reduced tonic currents in KO VB thalamic neurons. (*A Left*) The current recorded from a thalamic VB neuron from a WT mouse before and after application of 20 μM SR95531. SR95531 abolished spontaneous IPSCs and also induced a positive shift in the holding current due to blockade of a tonic inward current. (*A Right*) The all-points histograms corresponding to the 30-sec traces. The black and gray histograms illustrate the holding current in the absence and presence of SR95531, respectively. The dashed lines represent best-fit curves to a single Gaussian distribution. SR95531 caused a rightward shift and reduced the width of the all-point distribution. (*B Left*) The current recorded from a KO mouse VB neuron before and after application of 20 μM SR95531. SR95531 abolished spontaneous IPSCs without causing a shift in the holding current. (*B Right*) The corresponding all-points histograms. (*C*) Summary data for the WT and KO mice show that thalamic VB neurons from KO mice have no tonic inhibition (n = 9 and 17 for WT and KO, respectively). (*D*) VB neurons from KO mice also were insensitive to currents elicited by 0.1 and 0.3 μM GBX (n = 7–12; *, $P <$ 0.05). Averaged data are expressed as mean ± SE. Published in Chandra et al. Proc. Nat. Acad. Sci. 2006 103: 15230-35..

APPENDIX D

CELLULAR INSENSITIVITY OF GABRA4 KO MICE TO GABOXADOL



Reduced tonic currents in KO VB thalamic neurons. (*A Left*) The current recorded from a thalamic VB neuron from a WT mouse before and after application of 20 μM SR95531. SR95531 abolished spontaneous IPSCs and also induced a positive shift in the holding current due to blockade of a tonic inward current. (*A Right*) The all-points histograms corresponding to the 30-sec traces. The black and gray histograms illustrate the holding current in the absence and presence of SR95531, respectively. The dashed lines represent best-fit curves to a single Gaussian distribution. SR95531 caused a rightward shift and reduced the width of the all-point distribution. (*B Left*) The current recorded from a KO mouse VB neuron before and after application of 20 μM SR95531. SR95531 abolished spontaneous IPSCs without causing a shift in the holding current. (*B Right*) The corresponding all-points histograms. (*C*) Summary data for the WT and KO mice show that thalamic VB neurons from KO mice have no tonic inhibition (n = 9 and 17 for WT and KO, respectively). (*D*) VB neurons from KO mice also were insensitive to currents elicited by 0.1 and 0.3 μM GBX (n = 7–12; *, $P < 0.05$). Averaged data are expressed as mean ± SE. Published in Chandra et al. Proc. Natl. Acad. Sci. 2006, 103: 15230-35.

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